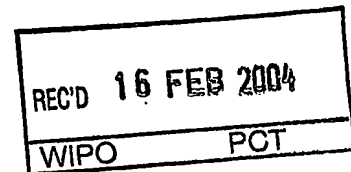




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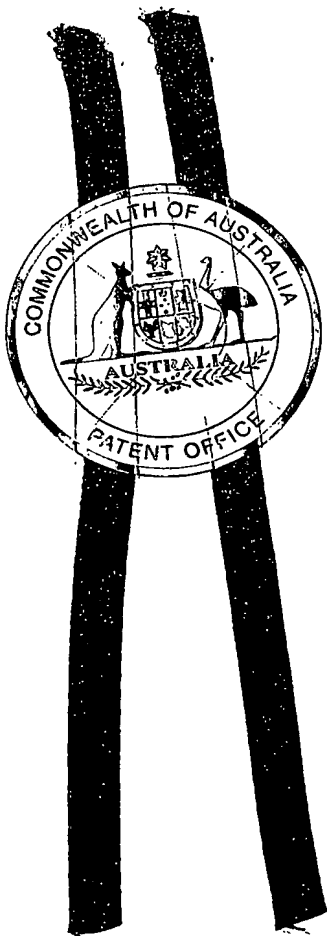
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**AUSTRALIA**

PATENTS ACT 1990

**PROVISIONAL SPECIFICATION**

***FOR THE INVENTION ENTITLED:-***

**A METHOD OF TREATING A MALIGNANCY IN A SUBJECT VIA DIRECT  
PICORNAVIRAL-MEDIATED ONCOLYSIS**

**The invention is described in the following statement:-**

A METHOD OF TREATING A MALIGNANCY IN A SUBJECT VIA DIRECT  
PICORNAVIRAL-MEDIATED ONCOLYSIS

Field Of The Invention

The present invention relates to the killing of abnormal cells utilising an  
5 echovirus. There is also described a method of screening cells to ascertain  
whether they are susceptible to treatment with the echovirus, as well as  
pharmaceutical compositions incorporating the echovirus. The invention finds  
veterinary use as well as broad application in the human medical field.

Background Of The Invention

10 The following discussion of the prior art is intended to present the  
invention in an appropriate technical context and allow its significance to be  
properly appreciated. Unless clearly indicated to the contrary, however,  
reference to any prior art in this specification should not be construed as an  
admission that such art is widely known or forms part of common general  
15 knowledge in the field.

Ovarian cancer is a leading cause of morbidity in the female population.  
Several malignancies arise from the ovary. Epithelial carcinoma of the ovary is  
one of the most common gynaecologic malignancies and the fifth most frequent  
cause of cancer death in women, with half of all cases occurring in women over  
20 age 65.

Approximately 5% to 10% of ovarian cancers are familial and 3 distinct  
hereditary patterns have been identified: ovarian cancer alone, ovarian and  
breast cancers, or ovarian and colon cancers. The most important risk factor for  
ovarian cancer is a family history of a first-degree relative (mother, daughter, or  
25 sister) with the disease. The highest risk appears in women with 2 or more first-

degree relatives with ovarian cancer. The risk is somewhat less for women with one first-degree and one second-degree (grandmother, aunt) relative with ovarian cancer. In most families affected with breast and ovarian cancer syndrome or site-specific ovarian cancer, genetic linkage has been found to the  
5 BRCA1 locus on chromosome 17q21. BRCA2, also responsible for some instances of inherited ovarian and breast cancer, has been mapped by genetic linkage to chromosome 13q12.

The lifetime risk for developing ovarian cancer in patients harbouring germ-line mutations in BRCA1 is substantially increased over the general  
10 population. Two retrospective studies of patients with germ-line mutations in BRCA1 suggest that these women have improved survival compared to BRCA1 negative women. When interpreting this data, it must be considered that the majority of women with a BRCA1 mutation probably have family members with a history of ovarian and/or breast cancer. Therefore, these women may have been  
15 more vigilant and inclined to participate in cancer screening programs that may have led to earlier detection. For patients at increased risk, prophylactic oophorectomy may be considered after the age of 35 if childbearing is complete. However, the benefit of prophylactic oophorectomy has not yet been established. A small percentage of women may develop a primary peritoneal  
20 carcinoma, similar in appearance to ovarian cancer, after prophylactic oophorectomy (Xiao, C. *et al.*, 2001). Epithelial carcinomas are the most common types of ovarian cancer. Stromal and germ cell tumors are relatively uncommon and comprise less than 10% of cases.

Ovarian cancer usually spreads via local shedding into the peritoneal  
25 cavity followed by implantation on the peritoneum, and via local invasion of

bowel and bladder. The highly lethal nature of this tumour is due to the absence of symptoms in women with early stages of this disease. The incidence of positive nodes at primary surgery has been reported as high as 24% in patients with stage I disease, 50% in patient with stage II disease, 74% in patients with stage III disease, and 73% in patients with stage IV disease. Tumor cells may also block diaphragmatic lymphatics. The resulting impairment of lymphatic drainage of the peritoneum is thought to play a role in development of ascites in ovarian cancer. Also, transdiaphragmatic spread to the pleura is common.

Prognosis in ovarian cancer is influenced by several factors, but multivariate analyses suggest that the most important favorable factors include younger age, good performance status, cell type other than mucinous and clear cell, lower stage, well differentiated tumor, smaller disease volume prior to any surgical debulking, absence of ascites, and smaller residual tumor following primary cytoreductive surgery. For patients with stage I disease, the most important prognostic factor is grade, followed by dense adherence and large-volume ascites. DNA flow cytometric analysis of stage I and stage IIA patients may identify a group of high-risk patients. Patients with clear cell histology appear to have a worse prognosis. Patients with a significant component of transitional cell carcinoma appear to have a better prognosis.

Although the ovarian cancer-associated antigen, CA 125, has no prognostic significance when measured at the time of diagnosis, it has a high correlation with survival when measured one month after the third course of chemotherapy for patients with stage III or stage IV disease (Rossmann, M.G. *et al.*, 2000). For patients whose elevated CA 125 normalizes with chemotherapy,

more than one subsequent elevated CA 125 is highly predictive of active disease, but this does not mandate immediate therapy.

Most patients have widespread disease at the time of diagnosis because ovarian cancer is often asymptomatic in its early stages as indicated above.

5   Partly as a result of this, yearly mortality in ovarian cancer is approximately 65% of the incidence rate. Long-term follow-up of suboptimally debulked stage III and stage IV patients reveals a 5-year survival rate of less than 10% even with platinum-based combination therapy. Nevertheless, early stages of the disease are curable in a high percentage of patients.

10   At present the treatment for late stage ovarian cancers involves a total abdominal hysterectomy, careful examination of serosal surfaces, and attempts to debulk all gross disease usually followed by combination chemotherapy that includes a platinum analogue. The survival rate is then between six to forty months, long term survival being less than ten percent.

15   There has been ongoing research with the aim of identifying molecules that are differentially expressed in benign and malignant ovarian tumours.

Ovarian carcinomas have been found to express the integrin  $\alpha_2\beta_1$  (Moser, T.L. *et al.*, 1996; Cannistra, S.A. *et al.*, 1995; Bartolazzi, A. *et al.*, 1993).  $\alpha_2\beta_1$  promotes metastatic dissemination of human ovarian epithelial carcinoma  
20   via specific binding interactions with type 1 collagen (Schiro, J.A. *et al.*, 1991; Cardarelli, P.M. *et al.*, 1992). Up-regulated surface-expression of integrin  $\alpha_2\beta_1$  has also previously been observed on human gastric carcinoma.

The interaction of  $\alpha_2\beta_1$  with type 1 collagen likely plays a critical role in peritoneal seeding as well as in metastasis, and over expression of  $\alpha_2\beta_1$  has  
25   been shown to induce metastatic properties in non-metastatic cells (Chan, B.M.

*et al.*, 1991). Blocking of  $\alpha_2\beta_1$  has been shown to largely inhibit adhesion of ovarian carcinomas by type 1 collagen.

Viruses capable of inducing lysis of malignant cells through their replication process are known as oncolytic viruses. Most oncolytic viruses require proliferation in the same species or cell lineage. Infection of a cell by a virus involves attachment and uptake into the cell which leads to or is coincidental with uncoating of the viral capsid, and subsequently replication within the cell (Fenner F., *et al.* The Biology of Animal Viruses. Academic Press. New York, 1974 Second Ed.)

10 Oncolytic viruses assessed for capacity to kill cancer cells have included the adenovirus subtype Egypt 101 virus which showed oncolytic activity in the HeLa uterine/cervix cancer cell line, mumps virus for treatment of gastric carcinoma, uterine carcinoma and cutaneous carcinoma, Newcastle Disease Virus (NDV), influenza virus for treatment of ovarian cancer, and adenovirus for  
15 treatment of for instance, cervical carcinoma (Nemunaitis J; 1999). Other reports have indicated that adenoviruses and attenuated poliovirus recombinants may have use in the treatment of malignant glioma cells (e.g. Andreansky S.S., 1996), and that reovirus shows lytic capability in human U87 glioblastoma cells and NIH-3T3 cells with an activated Ras signalling pathway  
20 (e.g. Strong J.E. *et al.*, 1998).

A vaccinia oncolysate has also been used in clinical trials to treat melanoma (Stage II) patients (Nemunaitis J., 1999). Modified, non-neurovirulent Herpes simplex viruses (HSV) have also been reported as showing promise for the treatment of brain tumours including intracranial melanoma, and  
25 subcutaneous human melanoma (Randazzo B.R., 1997), while adenovirus

infection has been reported to enhance killing of melanoma cells by the plant mitotoxin, saporin (Satyamoorthy K., 1997).

The receptor on target cells recognised by adenovirus differs for different adenovirus types. That is, adenovirus subgroups A, C, D, E and F for instance  
5 recognise the CAR receptor while Adenovirus type 5 (subgroup C), Adenovirus type 2 (subgroup C) and Adenovirus type 9 (subgroup D) recognise major histocompatibility class II molecule,  $\alpha_m\beta_2$  and  $\alpha_v$  integrins, respectively. The CAR receptor is known to be expressed on melanoma cell lines.

Heparan sulfate is recognised by Herpes simplex types 1 and 2 and human  
10 herpes virus 7, Adeno-associated virus type 2. The receptor for human Herpesvirus 7 is CD4 while Epstein-Barr virus recognises complement receptor Cr2 (CD21). Poliovirus type 1 and 2 recognise poliovirus receptor (Pvr) for cell adhesion while reovirus recognises sialic acid. Influenza A and B virus recognise the sialic acid N-acetyl neuraminic acid for cell adhesion. In contrast,  
15 influenza type C virus recognises the sialic acid 9-O-acetyl neuraminic acid. Vaccina virus recognises both epidermal growth factor receptor and heparan sulfate. Coxsackievirus A13, A15, A18 and A21 recognise ICAM-1 and the complement regulatory protein DAF (CD55) (see eg. Shafren D.R., et al 1997). DAF is also recognised by Enterovirus 70 (see eg. Flint SJ, et al (2000)  
20 Principles of Virology:molecular biology, pathogenesis and control. ASM Press, Washington).

Metastatic tumour spread is a pathological process associated with a series of adhesion/de-adhesion events coupled with regulated tissue degradation. Adhesion to and migration through the extracellular matrix is  
25 essential for tumour invasion.



Despite progress being made in the treatment of malignancies, the treatment of cancer including ovarian malignancies presents a major challenge for research and there remains the need for alternatives to existing therapy approaches.

5 Summary Of The Invention

It is an aim of the present invention to ameliorate one or more problems of the prior art or to at least provide a useful alternative.

The present invention stems from the recognition that significant killing of abnormal cells such as cancer cells expressing the integrin  $\alpha_2\beta_1$  may be  
10 achieved using an echovirus which recognises  $\alpha_2\beta_1$ .

Accordingly, in one aspect of the present invention there is provided a method of treating abnormal cells in a mammal comprising administering to the mammal an effective amount of an echovirus which recognises  $\alpha_2\beta_1$ , or a modified form thereof which recognises  $\alpha_2\beta_1$ , such that at least some of the cells  
15 are killed by the virus.

A single virus serotype which recognises  $\alpha_2\beta_1$  may be administered to the mammal or a plurality of different echoviruses which recognise  $\alpha_2\beta_1$  may be administered.

Hence, in another aspect of the present invention there is provided a  
20 method of treating abnormal cells in a mammal comprising administering to the mammal an effective amount of virus selected from the group of viruses consisting of echoviruses which recognise  $\alpha_2\beta_1$  and modified forms and combinations thereof that recognise  $\alpha_2\beta_1$ , such that at least some of the cells are killed by the virus.

The term "abnormal cells" for the purpose of the present invention is to be taken in a broadest sense to include malignant cells, the cells of any abnormal growth and any cells having abnormal upregulated expression of integrin  $\alpha_2\beta_1$ , relative to corresponding normal cells of the same cell type expressing their  
5 normal phenotype, whether the cells are cancer cells or not and whether the cells proliferate at an abnormal rate or not. Accordingly, the term encompasses pre-neoplastic and neoplastic cells, and non-cancer cells that may or may not ultimately develop into cancer cells. An abnormal growth may for instance be a benign or malignant tumour. The abnormal cells will usually be malignant cells.

10 Generally, the abnormal cells will have upregulated expression of  $\alpha_2\beta_1$  compared to surrounding tissue in which the abnormal cells are found. Hence, the virus will typically preferentially infect the abnormal cells due to the greater likelihood of contacting  $\alpha_2\beta_1$  on those cells. As such the virus may be used to effectively target the abnormal cells.

15 A method of the invention is particularly suitable for treating ovarian cancer in a patient or cancer that has metastasised from a primary ovarian tumour. However, the invention is not limited to the treatment of such cancers and methods described herein find application in the treatment of other cancers including melanoma and prostate tumours as well as breast cancer and  
20 colorectal cancer, and cancers that have spread therefrom to other sites in the body. For instance, the virus may be administered to melanoma cancer cells in areas of the body other than the skin of the mammal. Accordingly, methods of the invention extend to the treatment of a malignancy where the malignancy has metastasised to a site or tissue in the mammal not normally associated with  
25 infection by the virus used to treat malignancy.

Preferably, the malignancy is selected from the group consisting of ovarian cancer, melanoma, prostate cancer, gastric cancer, colorectal cancer, breast cancer and secondary cancers that have metastasised from these sites. Most preferably, the cancer is ovarian cancer.

5       Accordingly, in yet another aspect of the present invention there is provided a method of treating a malignancy in a mammal selected from the group consisting of ovarian cancer, melanoma, prostate cancer, breast cancer, colorectal cancer and cancer that have spread from an ovarian, melanoma, prostate cancer, breast cancer or colorectal cancer, comprising administering to  
10   the mammal an effective amount of virus selected from the group of viruses consisting of echoviruses which recognise  $\alpha_2\beta_1$  and modified forms and combinations thereof that recognise  $\alpha_2\beta_1$ , wherein at least some of the cells of the malignancy are killed by the virus.

      In yet another aspect of the present invention there is provided a method of  
15   treating a condition in a mammal selected from ovarian cancer and a malignancy that has spread from an ovarian cancer, comprising administering to the mammal an effective amount of virus selected from the group of viruses consisting of echoviruses which recognise  $\alpha_2\beta_1$  and modified forms and combinations thereof that recognise  $\alpha_2\beta_1$ , wherein at least some of the cells of  
20   the malignancy are killed by the virus.

      As such, in still another aspect of the present invention there is provided a method of treating a malignancy in a mammal selected from the group consisting of ovarian cancer, melanoma, prostate cancer, breast cancer, colorectal cancer and cancer that has spread from an ovarian, melanoma,  
25   prostate cancer, breast cancer or colorectal cancer, comprising administering to

the mammal an effective amount of an echovirus wherein at least some of the cells are killed by the virus.

The virus may also be used to screen abnormal cells to ascertain for instance whether the virus may be suitable for treating the mammal from which  
5 the cells were obtained or whether a different treatment protocol not involving the virus may be more beneficial to the mammal. Conversely, different viruses may be screened using samples of cells taken from the mammal in order to select the most appropriate virus for treating the mammal.

Accordingly, in another aspect of the invention there is provided a method  
10 of screening a sample of abnormal cells from a mammal to determine whether the cells are susceptible to echovirus induced cell death for evaluating the possibility of administering echovirus to the mammal to treat the abnormal cells in the mammal, comprising the steps of:

- (a) providing the abnormal cells;
- 15 (b) adding to the cells an effective amount of an echovirus which recognises  $\alpha_2\beta_1$ ;
- (c) incubating the abnormal cells in the presence of the echovirus for a period of time; and
- (d) determining whether the echovirus has infected and caused death of  
20 at least some of the abnormal cells.

In a further aspect of the present invention there is provided a method of screening a sample of cancer cells from a mammal selected from ovarian cancer cells, melanoma cells, prostate cancer cells, breast cancer cells, colorectal cancer cells and cells of a cancer that has spread from an ovarian,  
25 melanoma, prostate, breast or colorectal cancer, for determining whether the

cells are susceptible to echovirus induced cell death for evaluating the possibility of administering echovirus to the mammal to treat the cancer cells in the mammal, comprising the steps of:

- (a) providing the cancer cells;
- 5 (b) adding to the cancer cells an effective amount of an echovirus;
- (c) incubating the cancer cells in the presence of the virus for a period of time; and
- (d) determining whether the virus has infected and killed at least some of the cancer cells.

10 In a further aspect of the present invention there is provided a method of screening a sample of cancer cells from a mammal for determining whether the cells are susceptible to echovirus induced cell death for evaluating the possibility of administering echovirus to the mammal to treat the cancer cells in the mammal, wherein the cancer cells are selected from ovarian cancer cells and  
15 cancer cells that have spread from an ovarian cancer, and the method comprises the steps of:

- (a) providing the cancer cells;
- (b) adding to the cancer cells an effective amount of an echovirus which recognises  $\alpha_2\beta_1$ ;
- 20 (c) incubating the cancer cells in the presence of the echovirus for a period of time; and
- (d) determining whether the echovirus has infected and killed at least some of the cancer cells.

A virus may also be selected for use in a method of the invention by testing  
25 whether a given virus is capable of infecting and killing at least some of a

sample of the abnormal cells. In particular, the testing may involve screening a number of different viruses by incubating each virus with a sample of the abnormal cells respectively, and determining whether the cells are killed as a result of infection by the virus.

5       Hence, in still another aspect of the invention there is provided a method of screening an echovirus for ability to infect and kill abnormal cells expressing  $\alpha_2\beta_1$  obtained from a mammal for evaluating the possibility of administering echovirus to the mammal to treat the abnormal cells in the mammal, comprising the steps of:

- 10       (a) selecting an echovirus which recognises  $\alpha_2\beta_1$ ;  
      (b) incubating the echovirus with a sample of the abnormal cells for a period of time; and  
      (c) determining whether the echovirus kills at least some of the abnormal cells.

15       In yet another aspect of the present invention there is provided a method of screening a virus for ability to infect and kill cancer cells obtained from a mammal for evaluating the possibility of administering the virus to the mammal to treat the cancer cells in the mammal, wherein the cancer cells are selected from ovarian cancer cells, melanoma cells, prostate cancer cells, breast cancer  
20       cells and colorectal cancer cells and cells obtained from a cancer that has spread from an ovarian, melanoma, prostate, breast or colorectal cancer, and the method comprises the steps of:

- (a) selecting an echovirus;  
      (b) incubating the echovirus with a sample of the cancer cells for a period  
25       of time; and

(c) determining whether the echovirus killed at least some of the cancer cells.

The method may also comprise the step of comparing the ability of the selected virus to infect and cause the death of the cells with that of another virus  
5 subjected to steps (b) and (c) above utilising another sample of the cells.

Death of the cells will typically result from infection of the cells by the virus, and may be caused by either lysis of the cells due to intracellular replication of the virus or by infection triggering apoptosis most likely as a result of the activation of cellular caspases.

10 Once lysed, the cytosolic contents of infected cells may spill from the ruptured plasma membranes, and antigens including cell surface antigens capable of eliciting an immune response to the abnormal cells may be released. Hence, treatment of abnormal cells in a mammal in accordance with a method of the invention may provide a boost to the immunity of the mammal against the  
15 abnormal cells.

Accordingly, in another aspect of the invention there is provided a method of inducing an immune response in a mammal comprising infecting abnormal cells in the mammal with an echovirus whereby lysis of at least some of the cells is caused with release of antigens therefrom for generation of the immune  
20 response against the abnormal cells and wherein the virus recognises  $\alpha_2\beta_1$ .

In yet another aspect of the present invention there is provided a method of inducing an immune response in a mammal against ovarian cancer cells or cancer cells that have spread from an ovarian cancer, comprising infecting the ovarian cancer cells in the mammal with an echovirus whereby lysis of at least

some of the cells is caused with release of antigens therefrom for generation of the immune response.

Generally, the virus will be provided in the form of a pharmaceutical composition for use in a method of the invention. As such, in a yet further  
5 aspect there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier together with an echovirus for administration to a mammal in a method of the invention.

In another aspect of the invention there is provided the use of an echovirus in the manufacture of a medicament for treating abnormal cells expressing  $\alpha_2\beta_1$   
10 in a mammal, wherein the virus recognises  $\alpha_2\beta_1$  and is capable of killing at least some of the cells.

In another aspect of the present invention there is provided the use of an echovirus in the manufacture of a medicament for treating a malignancy selected from the group consisting of ovarian cancer, melanoma, prostate  
15 cancer, breast cancer, colorectal cancer, and cancer that has spread from an ovarian cancer, melanoma, prostate cancer or colorectal cancer, wherein the virus is capable of killing at least some of the cells of the malignancy.

Typically, the echovirus utilised in accordance with the invention will be an echovirus selected from the group consisting of echovirus EV1, Echovirus EV8  
20 and Echovirus EV22.

While the virus will usually be a common animal echovirus the invention is not limited thereto and a recombinant virus engineered to be capable of infecting and killing the abnormal cells, or a virus that has otherwise been modified to enhance its ability to infect and kill the cells, may be utilised.



Moreover, the same virus may be administered to the mammal during different treatment courses. Preferably, however, different viruses are used for different treatment courses to avoid or lessen the potential effect of any immune response to the previous virus administered. The virus may for instance be  
5 administered topically, intratumourally or systemically to the mammal.

The mammal may be any mammal suffering from a malignancy and in need of treatment. Preferably, the mammal will be a human being.

In still another aspect of the present invention there is provided a delivery device impregnated with an echovirus for application to an affected site of a  
10 mammal for treating the mammal in accordance with a method as described herein.

A method of the invention may be used as an adjunct to conventional cancer treatment or as a treatment in the absence of other therapeutic treatments. In particular, a method of the invention may be utilised where  
15 conventional treatment is not suitable or practical, or in the instance where excision of abnormal cells may leave scarring or disfigurement which is unacceptable to the patient, particularly from the patient's face such as from their nose or lip. Alternatively, the virus may be administered to the patient prior to and/or immediately after excision of the relevant abnormal cells.

20 Accordingly, the instant methods provide an alternative therapeutic treatment that may be used both following diagnosis of early stage and latter stage malignancy, and which further finds application in the killing of abnormal cells prior to and remaining after surgery.

Using protocols as described herein the skilled addressee will be able to  
25 readily select a suitable virus for use in the methods of the invention, and

determine which abnormal cells are susceptible to infection leading to the death of the cells.

The features and advantages of the invention will become further apparent from the following description of preferred embodiments of the invention.

5 Brief Description Of The Accompanying Drawings

Figure 1: Flow cytometric analysis of the levels of surface expressed ICAM-1, CAR, DAF and  $\alpha_2\beta_1$  on the surface of breast cancer cells. The breast cancer cells were incubated with R-phycoerythrin-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse immunoglobulin in the presence or absence of corresponding  
10 monoclonal antibodies specific for these receptors. The geometric mean of the conjugate sample was subtracted from the geometric mean of the enterovirus receptor sample revealing the relative level of expression of the receptor.

Figure 2: Lytic infection of breast cancer cells by the enteroviruses CAV21, CVB3, EV1, EV7 and PV1. Fifty percent endpoint titres were calculated  
15 and oncolysis was considered significant if the TCID<sub>50</sub>/ml endpoint was 10<sup>4</sup> or greater.

Figure 3: Flow cytometric analysis of the levels of surface expressed ICAM-1, CAR, DAF and  $\alpha_2\beta_1$  on the surface of colorectal cancer cells. The colorectal cancer cells were incubated with R-phycoerythrin-conjugated F(ab')<sub>2</sub>  
20 fragment of goat anti-mouse immunoglobulin in the presence or absence of corresponding monoclonal antibodies specific for these receptors. The geometric mean of the conjugate sample was subtracted from the geometric mean of the enteroviral receptor sample revealing the relative level of expression of the receptor.

Figure 4: Lytic infection of colorectal cancer cells by the enteroviruses CAV21, CVB3, EV1, EV7 and PV1. Fifty percent endpoint titres were calculated and oncolysis was considered significant if the TCID<sub>50</sub>/ml endpoint was 10<sup>4</sup> or greater.

5        Figure 5: Flow cytometric analysis of the levels of surface expressed ICAM-1, CAR, DAF and  $\alpha_2\beta_1$  on the surface of the prostate or pancreatic cancer cells. The prostate or pancreatic cancer cells were incubated with R-phycoerythrin-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse immunoglobulin in the presence or absence of corresponding monoclonal antibodies specific for  
10 these receptors. The geometric mean of the conjugate sample was subtracted from the geometric mean of the enteroviral receptor sample revealing the relative level of expression of the receptor.

Figure 6: Lytic infection of prostate and pancreatic cancer cells by the enteroviruses CAV21, CVB3, EV1, EV7 and PV1. Fifty percent endpoint titres  
15 were calculated and oncolysis was considered significant if TCID<sub>50</sub>/ml endpoint was 10<sup>4</sup> or greater.

Figure 7: Flow cytometric analysis of the levels of surface expressed ICAM-1, CAR, DAF and  $\alpha_2\beta_1$  on the surface of ovarian cancer cells. The ovarian cancer cells were incubated with R-phycoerythrin-conjugated F(ab')<sub>2</sub> fragment of  
20 goat anti-mouse immunoglobulin in the presence or absence of corresponding monoclonal antibodies specific for these receptors. The geometric mean of the conjugate sample was subtracted from the geometric mean of the enteroviral receptor sample revealing the relative level of expression of the receptor.

Figure 8: Lytic infection of ovarian cancer cells by the enteroviruses  
25 CAV21, CVB3, EV1, EV7 and PV1. Fifty percent endpoint titres were calculated

and oncolysis was considered significant if the TCID<sub>50</sub>/ml endpoint was 10<sup>4</sup> or greater.

Figure 9A: Photomicrographs of ovarian cancer cell monolayers infected for 72 hours with a 10<sup>-1</sup> dilution of EV1. At this viral input multiplicity, all cell lines displayed significant levels of oncolysis by EV1 (right) excluding the cell line A2780.

Figure 9B: Photomicrographs of ovarian cancer cell monolayers infected for 72 hours with a 10<sup>-1</sup> dilution of EV1. All cell lines displayed significant levels of oncolysis by EV1 (right) excluding the cell line SKOV-3.

Figure 10: Lytic infection of ovarian cancer cells with EV1. Seven of the ten cell lines are considered to be susceptible to oncolysis by EV1. Oncolysis was considered to be significant if a viral titre (TCID<sub>50</sub>/ml) was calculated to be 10<sup>4</sup> or greater.

Figure 11: EV1 binding inhibited in the presence of anti-  $\alpha_2\beta_1$ . Binding of [<sup>35</sup>S]-methionine labeled EV1 to ovarian cancer cell lines in the presence and absence of either anti- $\alpha_2\beta_1$  or anti-DAF MAbs. Levels of [<sup>35</sup>S]-methionine labeled virus bound was determined by liquid scintillation counting on a 1450 Microbeta TRILUX (Wallac, Finland).

Figure 12: Lytic infection of the ovarian cancer cell lines OWA-42 and IGROV-1 by EV1 in the presence or absence of anti-  $\alpha_2\beta_1$ MAb. 72 hours post infection the cells preincubated with the anti-  $\alpha_2\beta_1$ MAb remained completely protected. Cell survival was determined by staining with crystal violet methanol solution.

Figure 13: Lytic infection of OWA-42 ovarian cancer cell monolayers by EV1 in the presence or absence of anti -  $\alpha_2\beta_1$  MAb. Photomicrographs were

taken at 24, 48 and 72 hours post infection demonstrating the complete protection of the cells from EV1 infection due to the monoclonal antibody blockade of the  $\alpha_2\beta_1$  receptor.

Figure 14: DOV13 ovarian cancer cells were cultured within the ring insert  
5 and HeL cells (human fibroblast cells) were cultured in the outer ring. Post infection with EV1 the viable cells were stained with crystal violet methanol solution. EV1 specifically infected the ovarian cancer cells while the HeL cells remained healthy.

Figure 15: Flow cytometric analysis of the level of surface expressed  $\alpha_2\beta_1$   
10 on the melanoma cell line SkMel28. SkMel28 cells were incubated with R-phycoerythrin-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse immunoglobulin in the presence or absence of anti-  $\alpha_2\beta_1$ . The geometric mean of the conjugate sample was subtracted from the geometric mean of the sample determining the shift and thus the expression of the receptor. Significant  $\alpha_2\beta_1$  expression is  
15 demonstrated due to the shift in geometric mean.

Figure 16: Binding of [<sup>35</sup>S]-methionine labeled EV1 to SkMel28 melanoma cells in the presence and absence of either anti- $\alpha_2\beta_1$  or anti-DAF MAbs. Levels of [<sup>35</sup>S]-methionine labeled virus bound was determined by liquid scintillation counting on a 1450 Microbeta TRILUX (Wallac, Finland).  $\alpha_2\beta_1$  blockade resulted  
20 in significant inhibition of EV1 binding. Results are expressed as the mean of triplicate samples  $\pm$  standard error.

Figure 17: Lytic infection of SkMel28 melanoma cells with EV1. Cell survival was determined by crystal violet methanol solution. Significant lysis can be observed.

Detailed Description of Preferred Embodiments of the Invention

To determine whether a virus is capable of infecting and causing death of cells of a tumour, a biopsy may be taken from the tumour and a preparation of cells prepared using conventional techniques prior to: (i) confirming virus  
5 receptor cell surface expression and (ii) challenging the cells with the virus and monitoring the cells for infection and cell death over a predetermined incubation period, typically about 2 days although this may vary depending on the virus used. A number of viruses may be screened in this way simultaneously utilising different aliquot's of the prepared malignant cells, the virus showing the greater  
10 degree of infectivity and cell death may then be selected for administration to the subject from whom the biopsy was taken. Similarly, different malignant cell preparations from biopsies taken from different sources may be employed in an assay using a specific virus. The biopsies may be taken from different sites of a single individual or from a number of individuals.

15 A virus used in a method as described herein will desirably cause few or only minor clinical symptoms in the recipient. Such viruses are readily obtainable from commercial sources well known to the skilled addressee and can be screened for their effectiveness in the instant methods in the manner described above. Desirably, the virus will normally be an echovirus selected  
20 from the group consisting of Echovirus EV1, Echovirus EV7 and Echovirus EV22. Each of these viruses recognise  $\alpha_2\beta_1$  for cell infectivity. EV1 has for instance been associated with mild upper respiratory illnesses and also pleurodynia (Fields B. N. et al, 2000; McCracken A. W. et al, 1969).

The expression of  $\alpha_2\beta_1$  is believed to be upregulated on ovarian  
25 carcinomas due to the prevalent collagen I matrix it encounters in the

mesothelial . Numerous malignant melanomas have also been shown to express upregulated levels of  $\alpha_2\beta_1$  (Kramer R. H. and Marks N, 1989; Ramos D. M. et al, 1990). EV1 and collagen attach to  $\alpha_2\beta_1$  using different residues in domain I of the  $\alpha_2$  subunit (Bergelson J. H., 1993). The itegrin  $\alpha_2\beta_1$  cannot  
5 simultaneously accommodate EV1 and collagen. However, the virus binds  $\alpha_2\beta_1$  with a 10-fold increase in affinity compared to collagen I (Xing L, 2002).

For the purpose of simply screening a given virus to ascertain whether it is capable of infecting and causing the death of malignant cells, malignant cell lines may be used for this purpose rather than primary malignant cells isolated  
10 from a biopsy.

The selected virus will preferably be injected directly into a number of sites on a malignant tumour in order to maximise the area for potential infection of the tumour by the virus. Normally, tissue surrounding the tumour will also be injected or otherwise treated with the virus given the possibility of malignant cells  
15 being present in the tissue. If the tumour is not detected until it is relatively advanced, surrounding tissue may be injected with the virus following surgical excision of the tumour itself.

Rather than being injected directly into a malignant tumour, the virus may be administered systemically by intravenous injection into the blood stream of  
20 the recipient at a location adjacent to the tumour site for delivery to the tumour. Similarly, the virus may be administered subcutaneously, intraperitoneally or for instance, intramuscularly if deemed appropriate. Generally, however, direct injection into the tumour is preferred given the possibility of the existence of antibodies specific for the virus and thereby the potential decreased efficacy of  
25 alternate such modes of virus delivery.

The virus may also be applied topically to tumours either alone or in combination with direct injection of the virus into the tumour. Topical treatment of the tumour may be achieved by dropwise application of a composition comprising the virus and a suitable pharmaceutically acceptable carrier for  
5 maintaining the integrity of the virus to allow for infection of the malignant cells by the virus, or by swabbing the tumour with an applicator impregnated with such a composition. The applicator may comprise a wad or pad of suitable material that has been dipped in the solution. In the case of treatment of melanoma on the skin, the virus may also be applied by way of a delivery means  
10 impregnated with the virus and which is adapted for being pressed against the malignant site to be treated to thereby deliver the virus to the malignant cells. The delivery device may also comprise a patch, wad or the like impregnated with the virus and which is further provided with an adhesive surface or surfaces such as in the case of a sticking plaster, for adhering to the skin surrounding the  
15 melanoma for thereby holding the virus in contact with the melanoma.

Generally, one or more small incisions will be made into the malignancy and/or surrounding tissue to provide a site of entry for the virus into same.

In the case of ovarian cancer, or cancer in the vicinity of an ovary, the echovirus may be delivered directly to the ovary or affected site using a catheter  
20 or other suitable application instrument via insertion of the catheter or selected instrument along the corresponding fallopian tube.

The carrier medium used for inoculating the recipient with the virus may be a fluid such as physiological saline, or any other conventionally known medium deemed appropriate such as commercially available gels suitable for  
25 pharmaceutical use and for administering the virus to the site of treatment.



The inoculant will generally contain from about  $1 \times 10^2$  to about  $1 \times 10^{10}$  plaque forming units per ml of the inoculant. Preferably, the inoculant will contain greater than about  $1 \times 10^5$  plaque forming units per ml of inoculant. The amount of inoculant administered to the patient may be readily determined by  
5 the attending physician or surgeon in accordance with accepted medical practice taking into account the general condition of the patient, the stage and location of the malignancy together with the overall size and distribution of the area to be treated with the virus. Typically, the patient will be treated with an initial dose of the virus and subsequently monitored for a suitable period of time  
10 before a decision is made to administer further virus to the patient pending factors such as the response of the patient to the initial administration of the virus and the degree of viral infection and malignant cell death resulting from the initial treatment.

Desirably, an individual will be treated with the virus over a period of time  
15 at predetermined intervals. The intervals may be daily or range from 24 hours up to 72 hours or more as determined appropriate in each circumstance. The same or a different virus may be administered each time to avoid or minimise the effect of any immune response to a previously administered virus, and a course of treatment may extend for one to two weeks or more as may be  
20 determined by the attending physician. Most preferably, virus to which the mammal has not previously been exposed or to which the mammal generates a relatively minor immune response as may be determined by standard techniques will be administered.

While readily available known echoviruses may be suitably employed in a  
25 method of the invention, a virus modified or engineered using conventional

techniques may also be utilised. For instance, a virus may be modified to employ additional cell adhesion molecules as cell receptors. For example, a virus may be modified using site-directed mutagenesis so that the peptide motif "RGD" is expressed on the viral capsid surface. The RGD motif is recognised  
5 by  $\alpha_v$  integrin heterodimers and this capsid modification may for instance allow the virus to bind the integrin  $\alpha_v\beta_3$ , a cell adhesion molecule which has been shown to be upregulated on melanoma lesions (Natalia P.G; 1997) as has  $\alpha_2\beta_1$  potentially leading to enhanced uptake of the virus by the target cell.

The invention will now be described with reference to a number of  
10 examples described below.

#### Example 1

##### *1.1. Cell Lines*

IGROV-1, A2780, DU145, PC3, AsPC-1, PANC-1, T47-D, MDA-MB361, MDA-MB453, MDA-MB231, and MCF-7 cancer cell lines were obtained from the  
15 Garvan Institute, Sydney, New South Wales, Australia. BT-20, MDA-MB157, SK-BR-3, ZR-75-1, HCT116, LIM2537, SW480, SW620, 2008, JAM, OVCA-429, OVCAR-3, OVHS-1, OWA-42, SKOV-3, and DOV13 cancer cell lines were obtained from Peter MacCullum Cancer Institute, Melbourne, Victoria, Australia. SkMel28 Cells were obtained from Dr Ralph, Department of Biochemistry and  
20 Molecular Biology, Monash University, Victoria, Australia. HeL cells were obtained from Margery Kennett, Entero-respiratory Laboratory, Fairfield Hospital, Melbourne, Victoria, Australia.

All cells were cultured in RPMI containing 2-5% Fetal Calf Serum (FCS) excluding BT-20 cells which were cultured in  $\alpha$ -MEM media, and SkMel28 and  
25 HeLa cells which were cultured in DMEM media. All cells used were routinely

checked for presence of mycoplasma by ELISA (Roche Molecular Systems, CA, USA).

## 1.2. Viruses

Coxsackievirus A21 (CAV21) prototype strain, Kuykendall;  
5 Coxsackievirus B3 (CVB3) prototype strain, Nancy; Echovirus (EV1) prototype strain, Farouk; Echovirus (EV7) prototype strain, Wallace; and Poliovirus 1 (PV1) prototype strain, Mahoney; were obtained from Dr Margery Kennett, Enter-respiratory Laboratory, Fairfield Hospital, Melbourne, Victoria, Australia. All viruses were propagated and titrated in HeLa cells.

## 10 1.3 Monoclonal Antibodies (MAbs)

The anti-DAF MAb VIIIA7, which recognizes the third SCR of DAF, was obtained from Dr T. Kinoshita, Osaka University, Osaka, Japan. The anti-CAR MAb RmcB was obtained from Dr. J. M. Bergelson, Dana Farber Cancer Institute, Boston, Massachusetts. The anti- $\beta$ 2-Microglobulin MAb 918 was  
15 obtained from Dr. P. Minor, NIBSC, Hertfordshire, England. The anti- $\alpha_2\beta_1$  MAb AK7, recognizing the  $\alpha_2$  subunit, and the control antibody anti-GPIV (platelet membrane glycoprotein) MAb PTA-1 were obtained from Professor Gordon Burns, Department of Medical Biochemistry and Cancer Research, University of Newcastle, NSW, Australia. The anti-ICAM-1 MAb IH4 was obtained from Dr  
20 Andrew Boyd from the Queensland Institute for Medical Research, Queensland, Australia.

## 1.4. Flow Cytometric Analysis

Enteroviral receptor surface expression on cancer cells was analysed by flow cytometry. Dispersed cells ( $1 \times 10^6$ ) were incubated on ice with the  
25 appropriate MAb (5 $\mu$ g/ml diluted in PBS) for 20 minutes. Cells were washed with

PBS and pelleted by centrifugation before resuspension in 100 $\mu$ l of 1:50 dilution of R-phycoerythrin-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse immunoglobulin (Dako, Australia). Cells were washed, pelleted and resuspended in PBS. Cell surface receptor expression was analysed using a  
5 FACStar Analyser (Becton Dickinson, Sydney, Australia).

### 1.5. Virus Infectivity Assay

Confluent monolayers of cancer cells in the appropriate media containing 1% fetal calf serum (FCS) in 96 well tissue culture plates were inoculated with 10-fold serial dilutions (100 $\mu$ l/well in triplicates) of CAV21, CVB3, EV1, EV7 or  
10 PV1 and incubated at 37°C in a 5% CO<sub>2</sub> environment for 72 hours. To determine cell survival plates were incubated with 100 $\mu$ l/well of crystal violet methanol solution (0.1% crystal violet, 20% methanol, 20% formaldehyde, phosphate buffered saline (PBS)) for 24 hours and washed in distilled water.

The endpoint of a limiting dilution assay is the dilution of virus that affects  
15 50% of test units. Statistical procedures were employed to calculate the endpoint using the Reed and Muench method. Endpoints were expressed as the 50% tissue culture infectious dose per millilitre (TCID<sub>50</sub>/ml).

Where cell monolayer pre-treatment with anti-receptor monoclonal antibodies was required, cells were incubated with 100 $\mu$ l of anti-  $\alpha_2\beta_1$  AK7  
20 (20 $\mu$ g/ml diluted in PBS) for 1 hour at 37°C. Cell monolayers were then inoculated in duplicate samples of appropriate viral dilution and incubated at 37°C in 5% CO<sub>2</sub> environment for 72 hours before staining as described above.

Photomicrographs were taken at 24, 48 or 72 hours at 100X magnification (Olympus IX-FLA) using an inverted microscope.

### 1.6 Virus Purification

Six well tissue culture plates containing confluent monolayers of DOV13 cells were inoculated with 500 $\mu$ l EV1 ( $10^8$  TCID<sub>50</sub>/ml) for 1 hour at 37°C. Unbound virus was removed by washing three times with methionine/cysteine free media (ICN Biomedical, Ohio, USA) and cell monolayers were incubated in 1.3ml of this media for a further 2 hours before addition of 300 $\mu$ Ci of [<sup>35</sup>S]-methionine trans-label (ICN Biomedical, Ohio, USA). Infected monolayers were incubated overnight at 37°C in a 5% CO<sub>2</sub> environment. Following three freeze/thaw cycles viral lysates were purified in a 5-30% sucrose gradient by velocity centrifugation for 95 minutes at 36, 000 rpm in a Beckman XL-90 ultracentrifuge (SW41ti Rotor). Fractions were collected from the bottom of each tube and monitored by liquid scintillation counting (Wallac 1450' Microbeta TRILUX, Finland) to locate 160S viral peak fraction used in viral binding assays.

### 1.7 Radiolabeled Virus Binding Assay

Binding assays were performed as cell suspensions. Approximately  $1 \times 10^6$  cells in 800 $\mu$ l of RPMI containing 1% bovine serum albumin (BSA) were incubated in the presence of 20 $\mu$ g/ml of MAb (anti- $\alpha_2\beta_1$  or anti-DAF diluted in PBS) for 1 hour at 4°C followed by the addition of 300 $\mu$ l ( $1 \times 10^6$ ) of [<sup>35</sup>S]-methionine labeled 160S EV1. After incubation at 4°C for 2 hours cells were washed four times with serum free media and cell pellets dissolved in 200 $\mu$ l 0.2M NaOH-1%SDS before the level of [<sup>35</sup>S]-methionine labeled virus bound was determined by liquid scintillation counting from triplicate samples. (Wallac 1450 Microbeta TRILUX, Finland). Results were expressed as means  $\pm$  SE.

*1.10 Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

[<sup>35</sup>S]-methionine labeled viral fractions were analysed by polyacrylamide gel electrophoresis (PAGE) and visualised by autoradiography. [<sup>35</sup>S]-methionine labeled 160S EV1 fractions were incubated with sample reducing buffer (250mM TRIS, 0.2g w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol and 0.01% w/v bromophenol blue, pH 6.8) for 10 minutes at 95°C denaturing the virion. Denatured 160S vial peak fractions were then separated on a 15% Tris-HCl precast gel (BIORAD Ready-Gel, CA, USA) in conjunction with a Benchmark prestained midrange protein ladder (GIBCO, USA) at 180V for 45 minutes. Visualisation of the major structural proteins and analysis of viral purity was by autoradiography on Hyperfilm MP (Amersham International, England) after 96 hour exposure.

Example 2

*2.1 Expression of Enterovirus Receptors on the Surface of Breast Cancer Cells*

To determine the relative expression levels of selected enteroviral cell surface receptors used by enteroviruses flow cytometric analysis was performed. The selected group of receptors consisted of ICAM-1 employed by CAV21; DAF employed by EV7, CAV21, CVB3; CAR used by CVB3; and integrin  $\alpha_2\beta_1$  used by EV1. Due to the unavailability of Mab against the PVR receptor, no expression levels of PVR were determined.

Nine breast cancer cell lines were analysed including BT-29, MCF-7, MDA-MB157, MDA-MB231, MDA-MB361, MDA-MB453, SK-BR-3, T47-D and ZR-75-

1. The cell lines were incubated with either anti-ICAM-1 (IH4), anti-CAR (RmcB), anti-DAF (VIII A7) or anti- $\alpha_2\beta_1$  (AK7).

ICAM-1 expression was significant in six of the nine lines while DAF appeared to be expressed at relatively low levels in all the cell lines. Moderate levels of CAR expression were evident on seven of the nine lines, while minimal levels of  $\alpha_2\beta_1$  expression were present on the surface of eight of the breast cancer lines (Figure 1).

## 2.2 Oncolysis of Breast Cancer Cells by Selected Enteroviruses

Lytic infectivity assays were performed on all nine of the breast cancer cell lines to determine their susceptibility to a select group of enteroviruses, CAV21, CVB3, EV1, EV7 and PV1 (Figure 2). A cell line was considered to be highly susceptible to oncolysis if the tissue culture infectious dose at a fifty percent endpoint per millilitre (TCID<sub>50</sub>/ml) was calculated to be  $10^4$  or greater.

CAV21 and CVB3 induced significant lysis in six of the nine breast cancer cell lines. In general breast cancer cells were not susceptible to lytic infection by the echoviruses EV1 and EV7 excluding one cell line T47-D which demonstrated considerable susceptibility to EV1. PV1 caused substantial oncolysis in eight of the nine breast cancer cell lines (Figure 2).

## 2.3 Expression of Enterovirus Receptor on the Surface of Colorectal Cancer Cells

Four cancers originating in the colorectal (HCT116, LIM2537, SW480 and SW620) were analysed for expression of ICAM-1, CAR,  $\alpha_2\beta_1$  and DAF by flow cytometry. Significant levels of ICAM-1 and DAF expression were observed on two of the cell lines. Moderate levels of CAR appeared to be expressed on all four lines, while significant levels of  $\alpha_2\beta_1$  expression were not observed (Figure 3).

#### 2.4 Oncolysis of Colorectal Cancer Cells by Selected Enteroviruses

CAV21, CVB3, EV1, EV7 and PV1 were titrated in all four colorectal cancer cell lines. Significant levels of oncolysis by CVB3 and PV1 were observed in all four cell lines (Figure 4). However significant cell lysis induced by CAV21 occurred in only one of the four cell lines (LIM2573), not surprisingly this cell line exhibited the highest level of ICAM-1 expression. Despite very low expression levels of  $\alpha_2\beta_1$  EV1 lytically infected three of the cell lines while all cells were refractile to EV7 infection.

#### 2.5 Expression of Enterovirus Receptors on the Surface of Prostate and Pancreatic Cancer Cells

Prostatic cancer cell lines including DU145 and PC3 and pancreatic cancer cell lines including AsPC-1 and PANC-1 were analysed for expression of ICAM-1, DAF, CAR and  $\alpha_2\beta_1$ . Significant levels of ICAM-1 was expressed on both of the prostatic cell lines and on one of the pancreatic lines. Moderate CAR and DAF expression was found on all four of the cell lines while  $\alpha_2\beta_1$  expression appeared to be minimal (Figure 5).

#### 2.6 Oncolysis of Prostate and Pancreatic Cancer Cells

The susceptibility of two prostate cancer cell lines and two pancreatic cancer cell lines to enteroviruses CAV21, CVB3, EV1, EV7 and PV1 were examined in microtitre plate lytic infections. The prostatic cancer cell lines were susceptible to all the viruses excluding EV7 in the case of DU145. PANC-1 was only infected by CAV21 and PV1, whereas the other pancreatic cancer cell line AsPC-1 exhibited oncolysis by all viruses excluding EV7 (Figure 6).



### *2.7 Expression of Enterovirus Receptors on the Surface of Ovarian Cancer Cells*

Ovarian cancer cell lines were examined for expression of enterovirus receptors ICAM-1, CAR, DAF and  $\alpha_2\beta_1$ . Nine cell lines were included; A2780, DOV13, IGROV-1, JAM, OVCA-429, OVHS-1, OWA-42, SKOV-3 and 2008.

5 Significant levels of ICAM-1 were expressed on two of the nine cell lines while moderate levels of CAR expression were present on six of the nine. DAF was expressed at high to moderate levels on all but one of the ovarian cancer cell lines. Eight of the nine ovarian cancer cell lines exhibited moderate to high level expression of  $\alpha_2\beta_1$  (Figure 7), with an additional ovarian cancer cell line  
10 (OVCAR-3) expressing significant levels of  $\alpha_2\beta_1$  (data not shown).

### *2.8 Oncolysis of Ovarian Cancer Cell Lines*

The oncolytic capacity of CAV21, CVB3, EV1, EV7 and PV1 was assessed in each of the nine ovarian cancer cell lines (Figure 8). CAV21 susceptibility was discovered on two of the nine cell lines while CVB3 caused  
15 significant lysis in seven of the nine lines. Ovarian cancers seemed particularly susceptible to echoviruses with EV7 causing death in four of the nine cancer cell lines and EV1 causing seven of the ten cell lines to lyse significantly upon infection (Figure 9A, 9B and 10). Vulnerability to PV1 was revealed across all nine ovarian cancer cell lines. Photomicrographs were taken of all ten lines  
20 infected with EV1 (Figures 9A and 9B) and a microtitre plate lytic infection of the ten ovarian cancer cell lines with EV1 can also be observed (Figure 10).

### *2.9 Binding of EV1 to Ovarian Cancer Cell Lines*

As ovarian cancer cell lines were highly susceptible to oncolysis by EV1 further investigations involving the nature of EV1 cell attachment was undertaken.

5        Cells were preincubated with either anti-  $\alpha_2\beta_1$  (AK7) or anti-DAF (VIII A7) monoclonal antibodies before radiolabeled EV1 was added to determine the involvement of these receptors in EV1 host cell binding.

Binding of EV1 was apparent on all ten of the cell lines tested. By blocking the  $\alpha_2\beta_1$  integrin with anti-receptor antibody cellular attachment of EV1  
10        was significantly inhibited. Blocking of the cell surface receptor DAF with the monoclonal antibody VIII A7 caused no significant inhibition of EV1 binding (Figure 11).

### *2.10 Antibody Blockade of $\alpha_2\beta_1$ Integrin Inhibits EV1 Infection of Ovarian Cancer Cell Lines*

15        In order to assess the function of  $\alpha_2\beta_1$  in EV1 infection, a lytic assay was performed where the cell monolayer was preincubated with anti-  $\alpha_2\beta_1$  (AK7) monoclonal antibody. OWA-42 and IGROV-1 ovarian cancer cell lines were analysed.

After 72 hours post virus infection the cell monolayers in the absence of  
20        MAb blockade were highly susceptible to EV1 lytic infection. Following MAb blockade of the  $\alpha_2\beta_1$  integrin there was no indication of oncolysis in the cell lines even at the lowest dilution of EV1 (Figure 12).

Photomicrographs were taken at 24, 48 and 72 hours post infection of the OWA-42 cell line (Figure 13).

#### *2.11 Non-cancerous Human Cells Not Susceptible to EV1 Infection*

An experiment was performed to examine the effect that EV1 has on non-cancerous human cells, determined by infecting human fibroblasts with EV1. Briefly, 6-well tissue culture plates were prepared with a tissue culture ring insert, DOV13 cells within the ring and HeL cells, human fibroblasts (obtained from CSL, Australia), in the outer ring incubated at 37°C until confluent monolayers were formed. The ring was removed and cells infected with EV1 overnight at 37°C. Viable cells were stained with crystal violet methanol solution.

Upon infection with EV1 the DOV13 ovarian cancer cells were lysed whereas the HeLa cells remained healthy (Figure 14) demonstrating the specific susceptibility of the ovarian cancer cells to EV1.

#### *2.10 Expression of $\alpha_2\beta_1$ on Melanoma Cell Line SkMel28*

Melanomas, cancer of the skin, are known to up regulate  $\alpha_2\beta_1$  expression. The melanoma cell line SkMel28 was examined for expression using flow cytometry. High levels of  $\alpha_2\beta_1$  expression were observed, however, a low background level of binding was exhibited by the control MAb (Figure 15).

#### *2.11 Binding of EV1 to SkMel28*

To further investigate the nature of EV1 attachment to surface expressed  $\alpha_2\beta_1$  on SkMel28 cells, radiolabeled virus binding assays were undertaken. The radiolabeled EV1 bound significantly to the malignant melanoma cell line with MAb blockade of  $\alpha_2\beta_1$  severely depleting the amount of EV1 bound (Figure 16).

## 2.12 Infectivity Assay of SkMel28 with EV1

A lytic infectivity assay was performed to determine the susceptibility of SkMel28 to EV1 infection. The malignant melanoma cell line displayed moderate oncolysis upon infection with EV1. The crystal violet stain was absorbed by cells not undergoing lytic infection where as the non-stained wells represent complete lysis of cell monolayers (Figure 17).

### Discussion

Ovarian cancer cell lines were found to be highly susceptible to lytic infections by EV1 with seven of the ten cell lines tested showing significant oncolysis. Further studies into the binding of EV1 to the ovarian cancer cell lines confirmed that  $\alpha_2\beta_1$  is the primary receptor used by EV1. The radiolabeled binding studies further indicated  $\alpha_2\beta_1$  , was required for virus binding and the MAb blocking assay revealed that by pre-treating susceptible ovarian cancer cells with an  $\alpha_2\beta_1$  monoclonal antibody (Mab), EV1 infection was completely inhibited. The DAF MAb VIIIA7 was also used in the binding assay as a negative control treatment to determine if DAF played a role in EV1 binding as it does with the enteroviruses CAV21 and CVB3. No significant blockage of EV1 binding occurred with anti-DAF MAb pre-treatment.

Co-culturing ovarian cancer cells with human fibroblasts followed by EV1 infection revealed that human fibroblast cells were not susceptible to EV1 infection even in an environment where the virus specifically lysed the ovarian cancer cells.

The effect of EV1 mediated oncolysis on a melanoma cell line was also investigated. The data revealed that  $\alpha_2\beta_1$  was up regulated on the surface of the SkMel28 melanoma cell line and that these cells were susceptible to EV1 lytic

infection. The binding of EV1 to the ovarian cancer cells was shown to be via  $\alpha_2\beta_1$  interactions as revealed by the radiolabeled binding assay.

The remaining cancer cell lines that were permissive for EV1 infection were colon cancer cell lines with three of the four cell lines highly susceptible as well as both prostate cancer cell lines. Both these cancer types may encounter the same extracellular matrix as ovarian cancer cells and hence, upregulate their  $\alpha_2\beta_1$  expression during metastasis through the extracellular matrix rich in collagen I encountered in the peritoneal surfaces.

Although the present invention has been described hereinbefore with reference to a number of preferred embodiments, the skilled addressee will understand that numerous modifications and variations are possible without departing from the scope of the invention.

DATED this 18<sup>th</sup> Day of December 2002.

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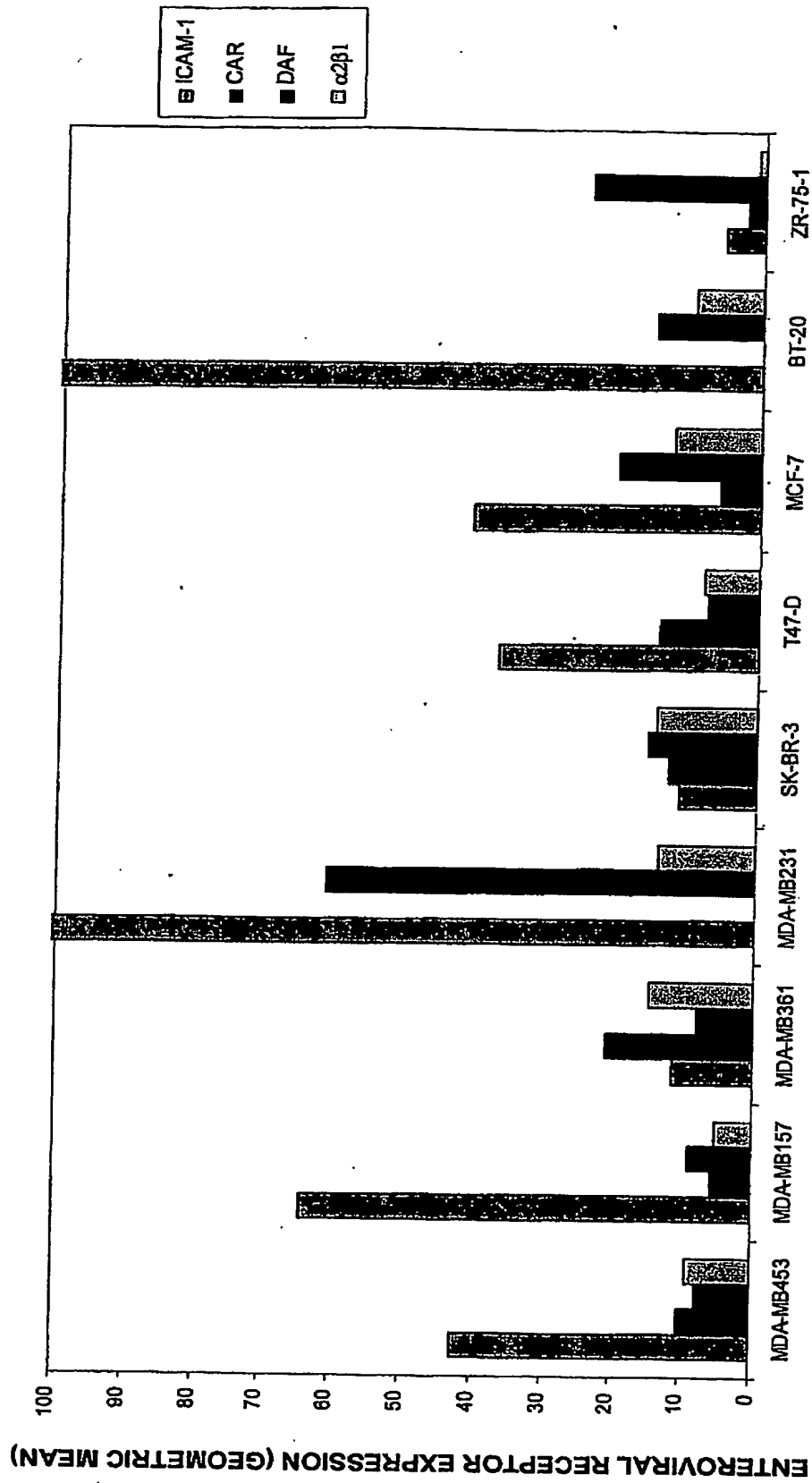


Fig. 1

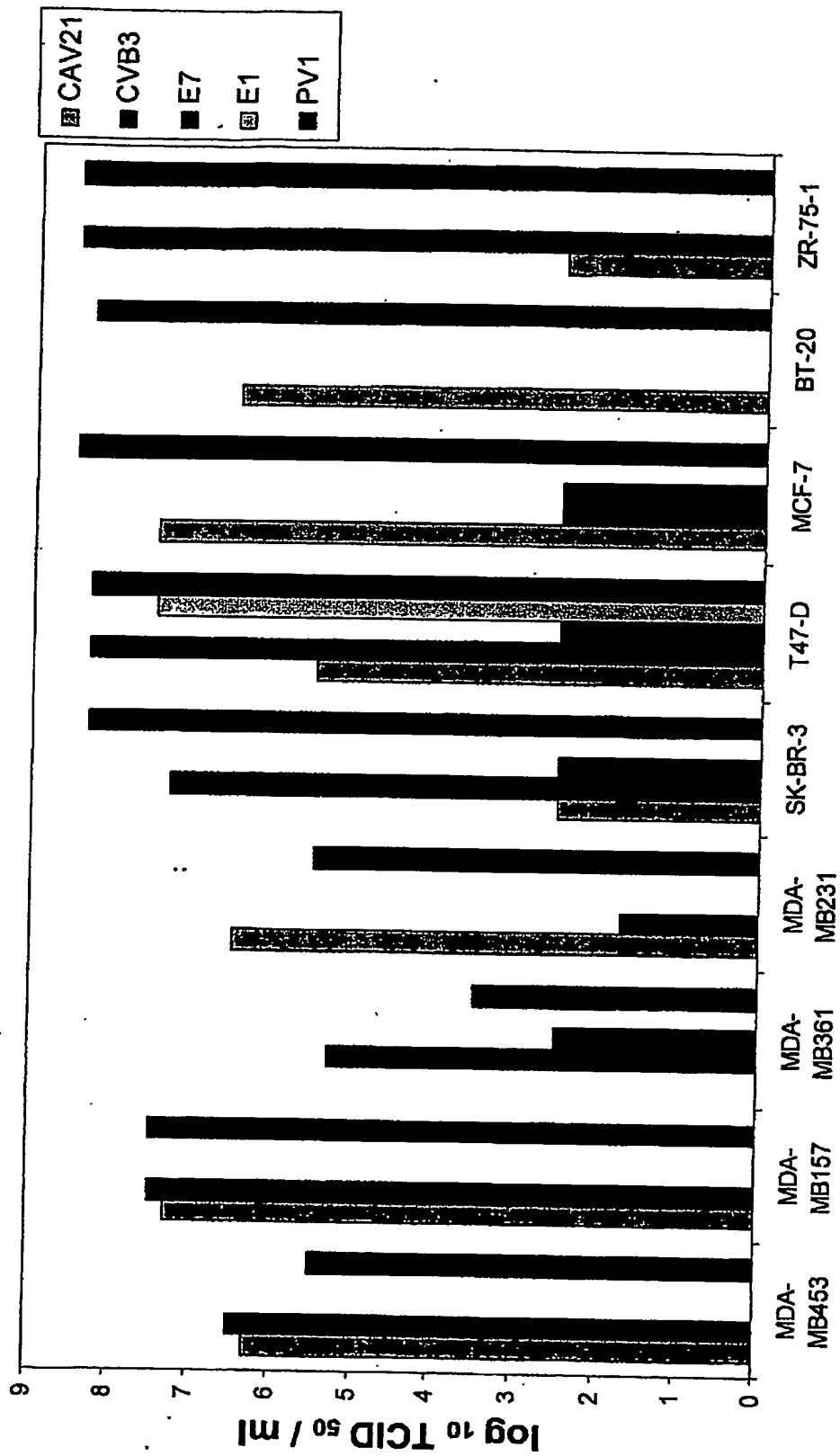


Fig. 2

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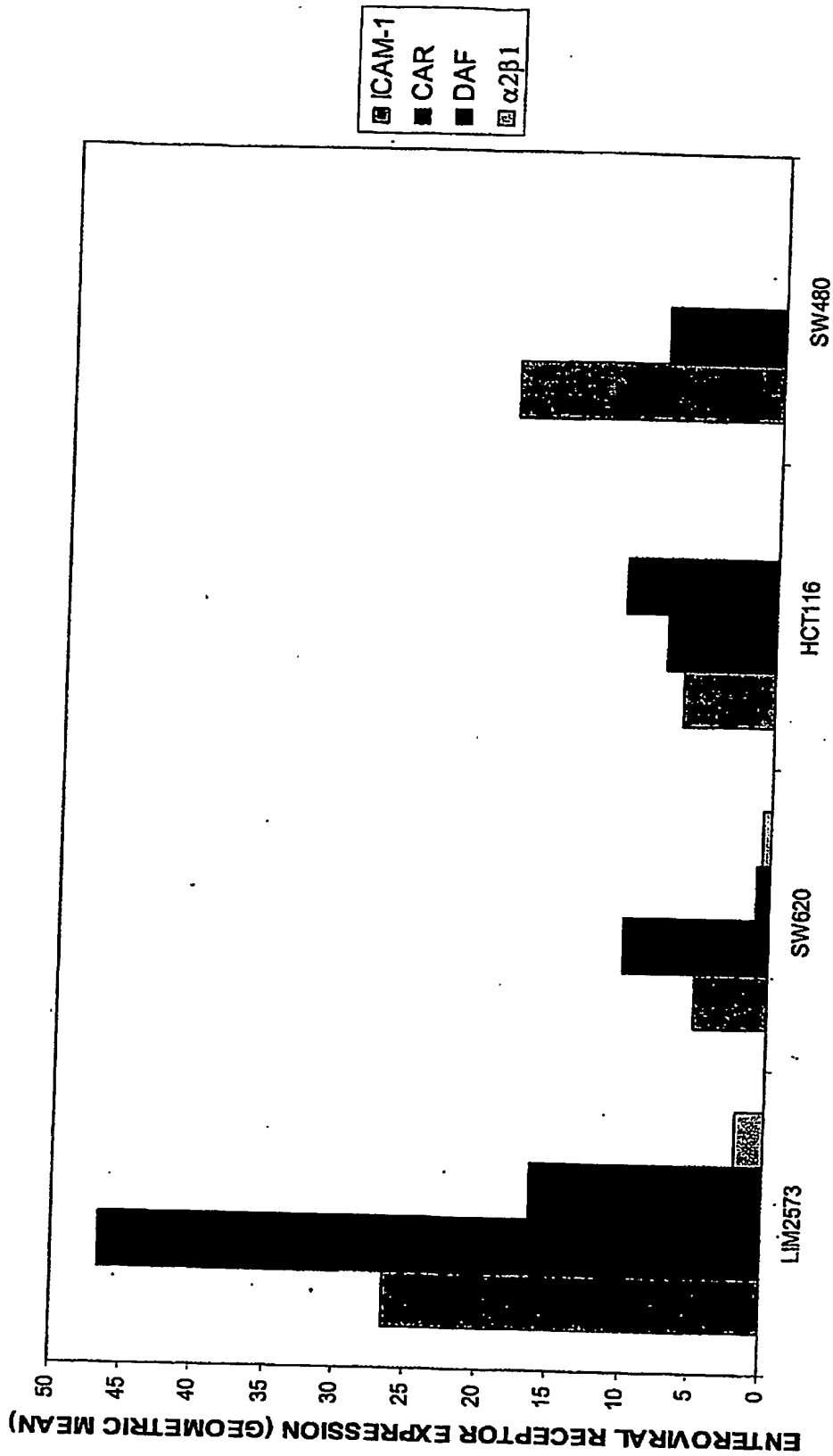


Fig. 3

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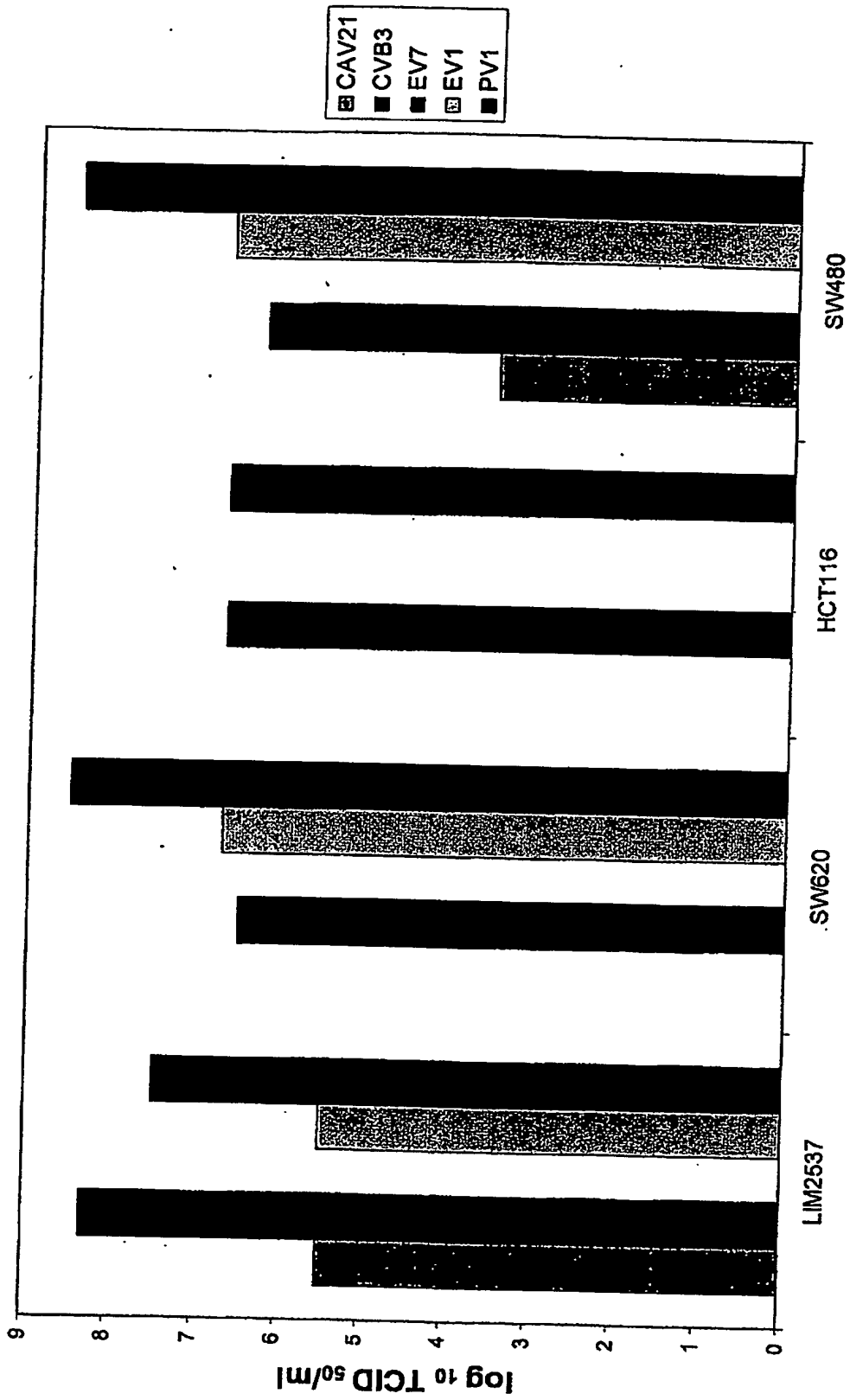


Fig. 4

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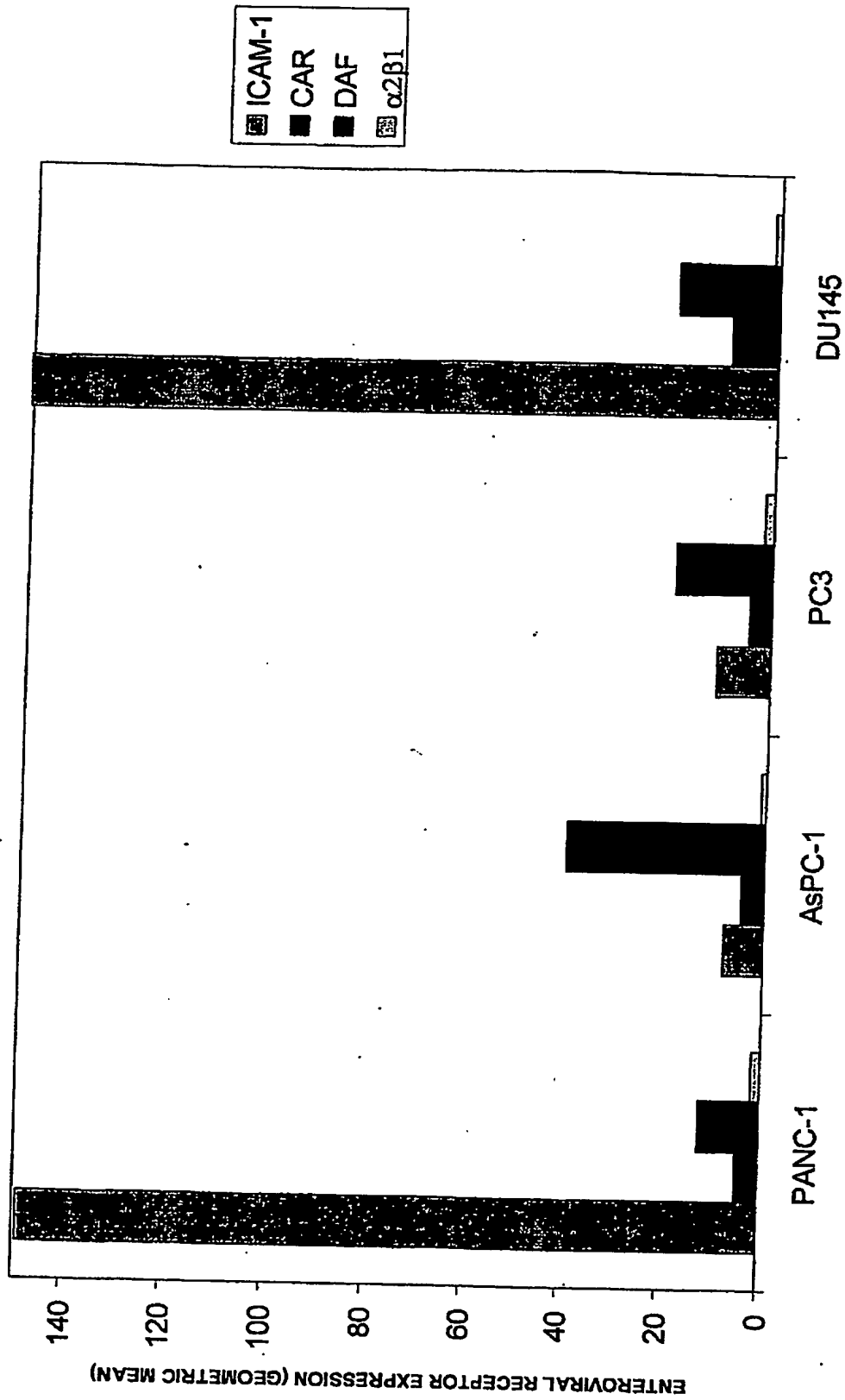


Fig. 5

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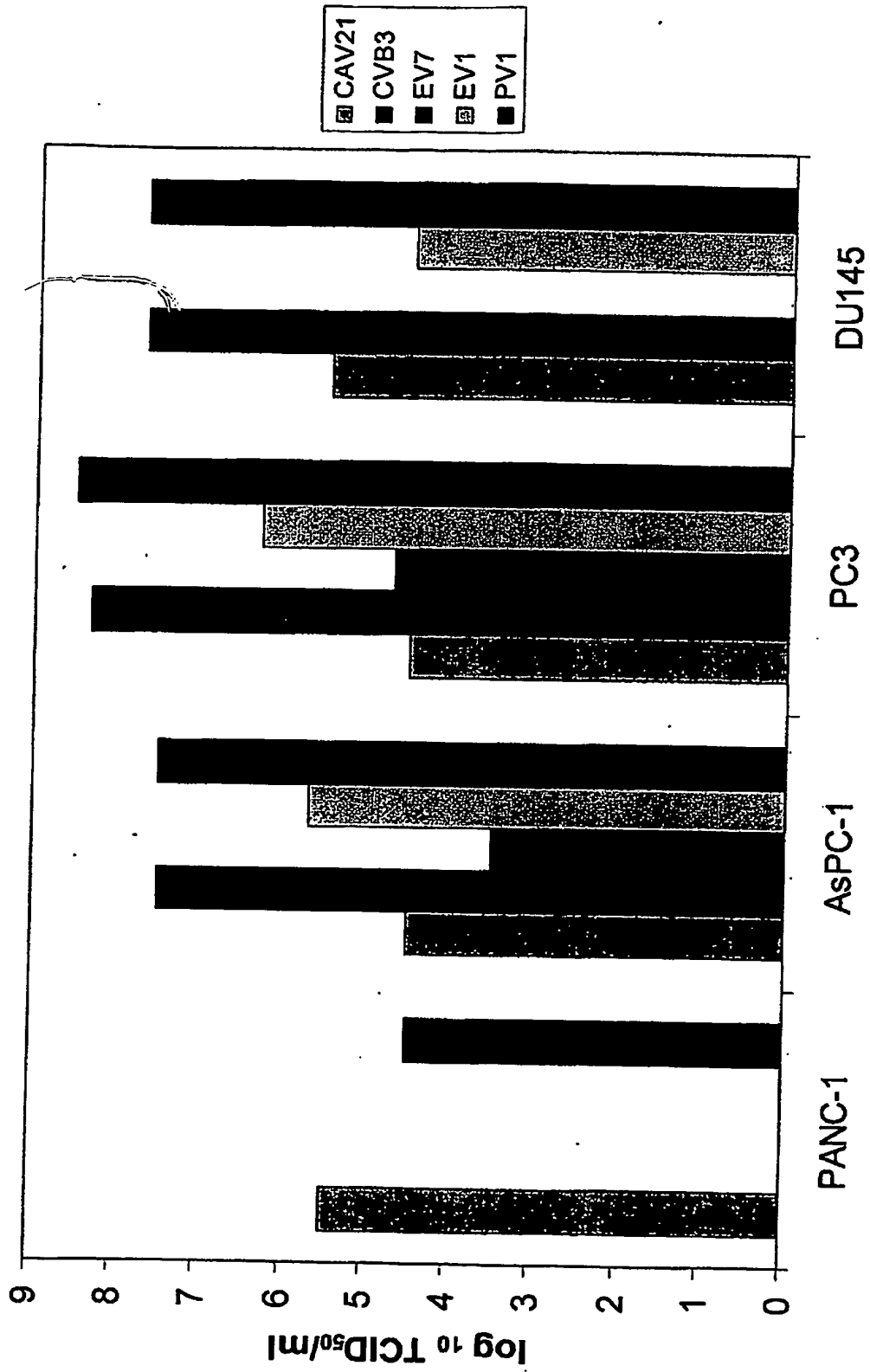


Fig. 6

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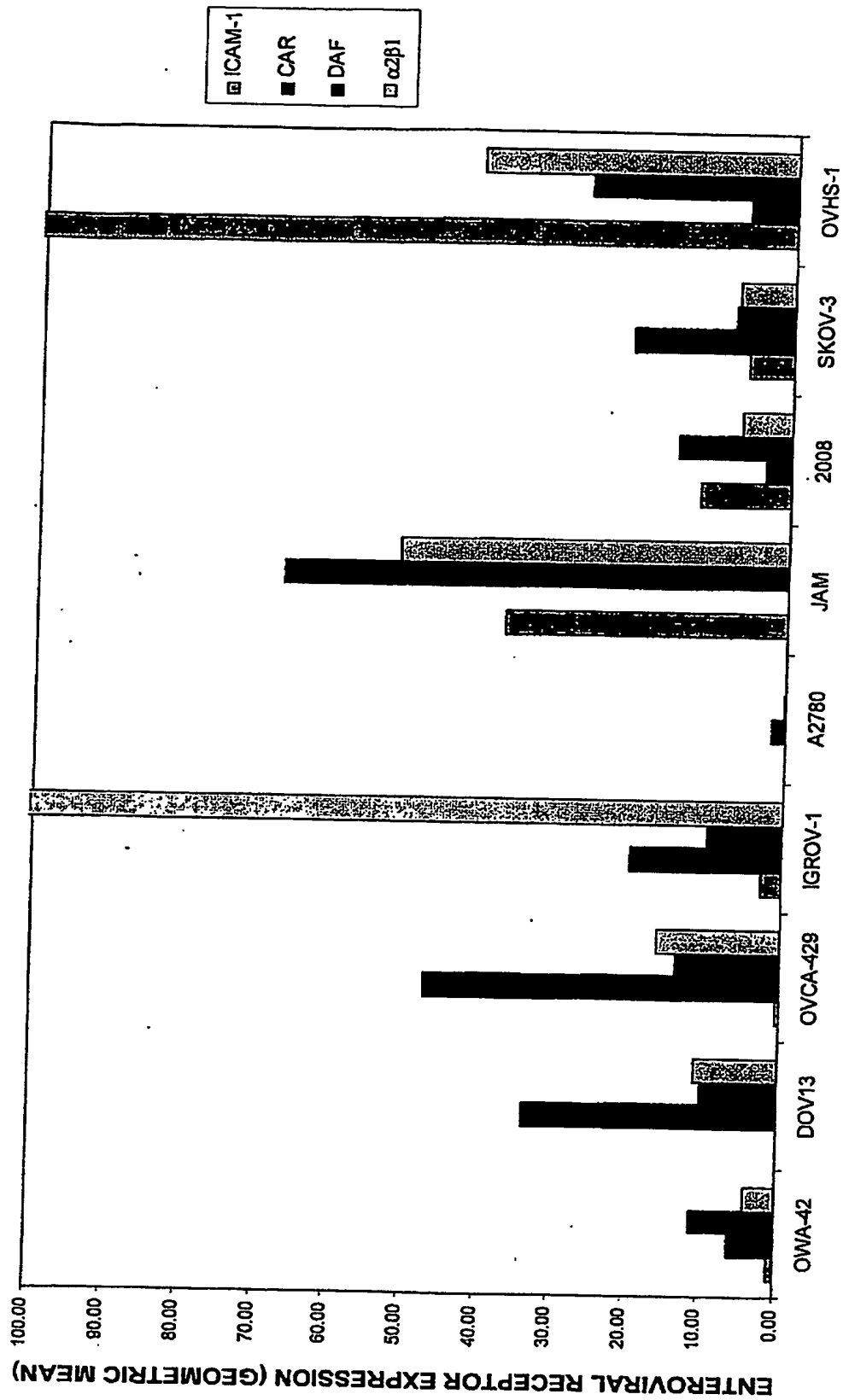


Fig. 7

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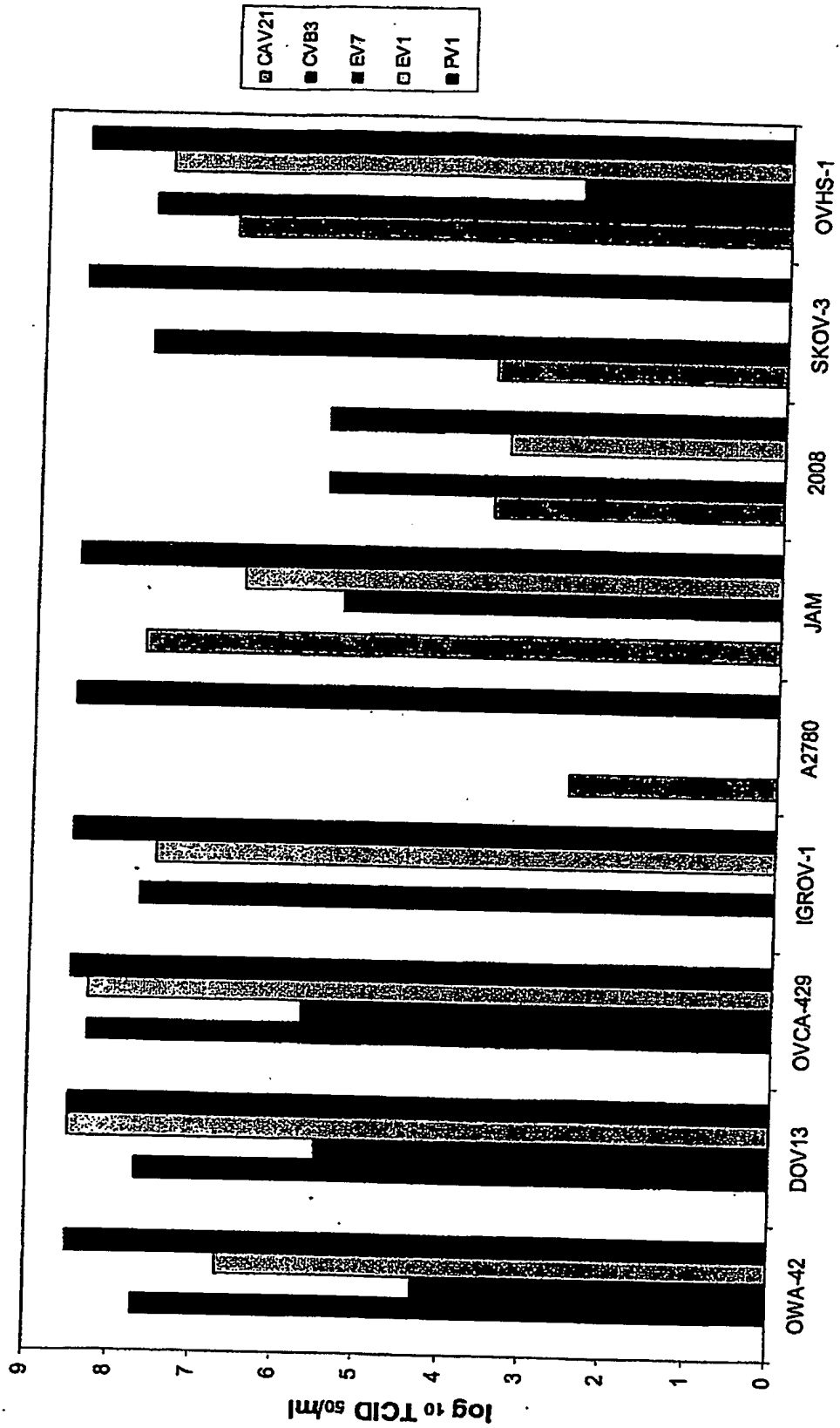


Fig. 8



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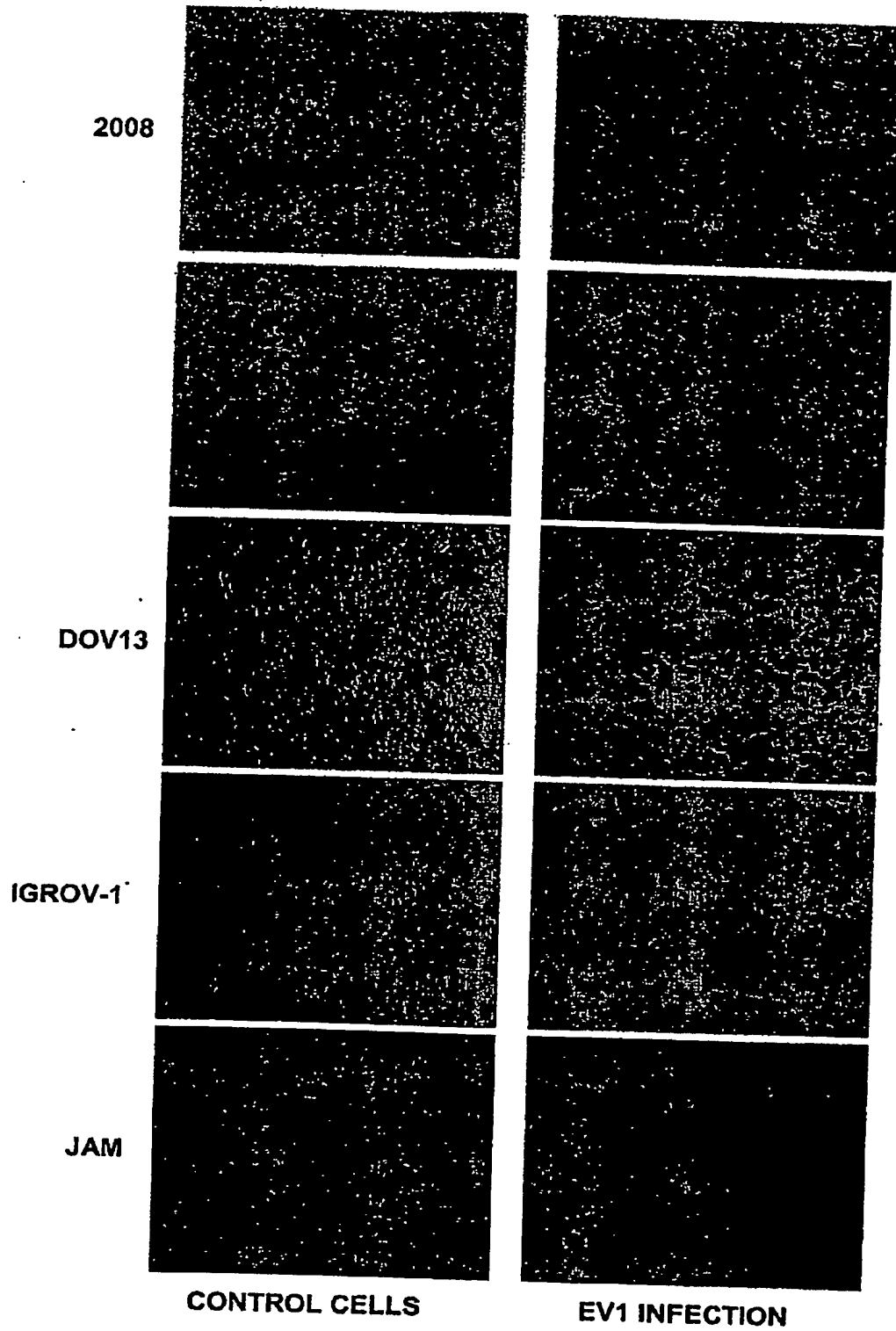


Fig. 9a

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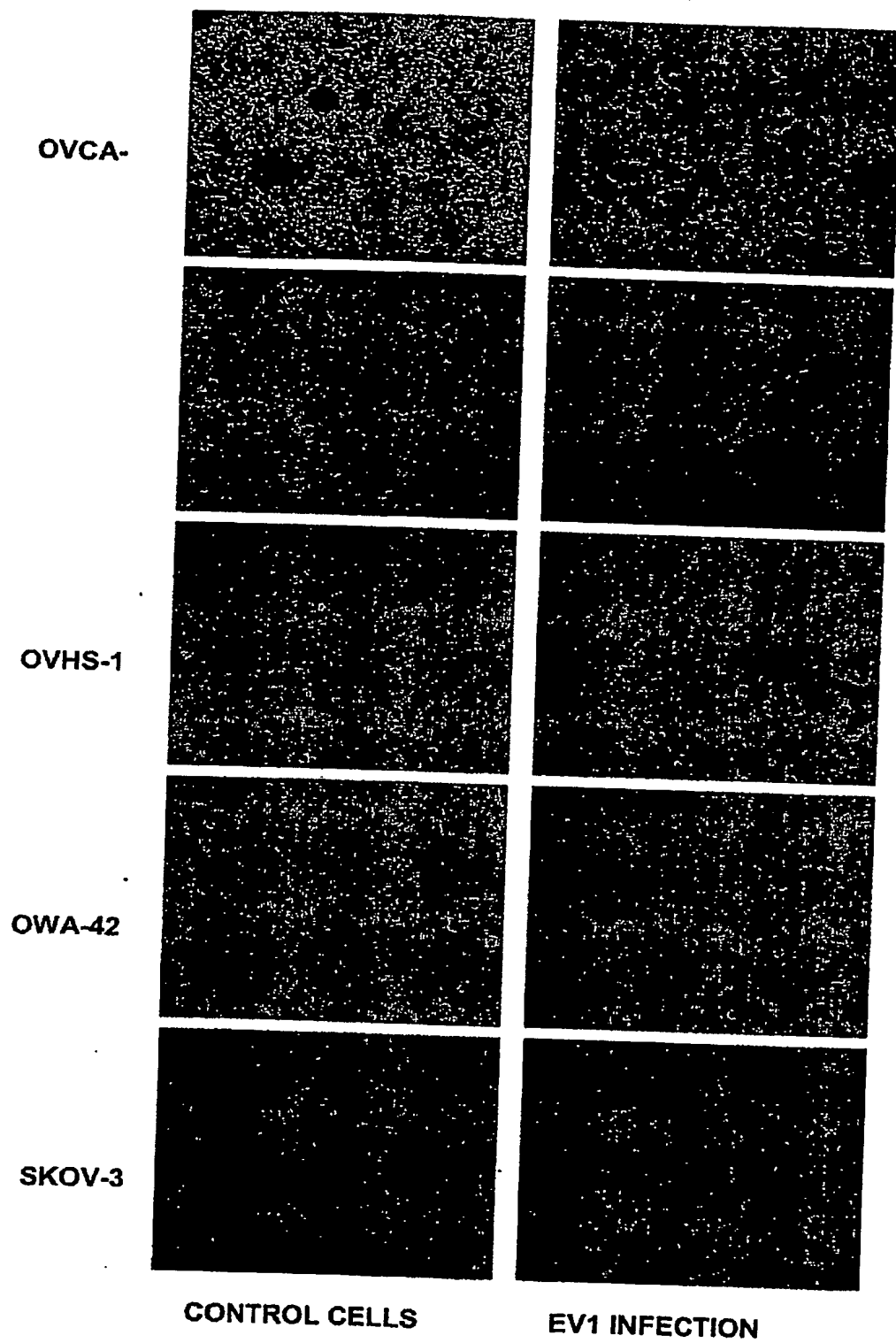


Fig. 9b

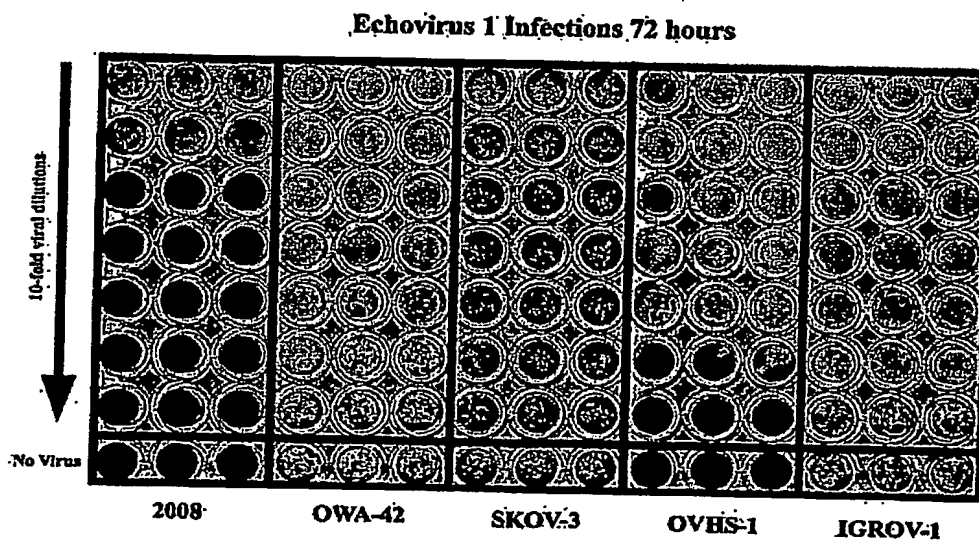
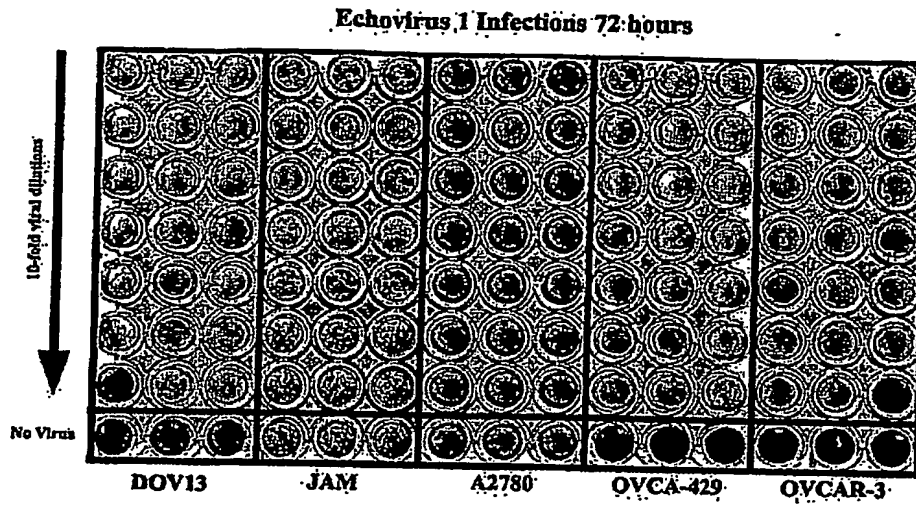


Fig. 10

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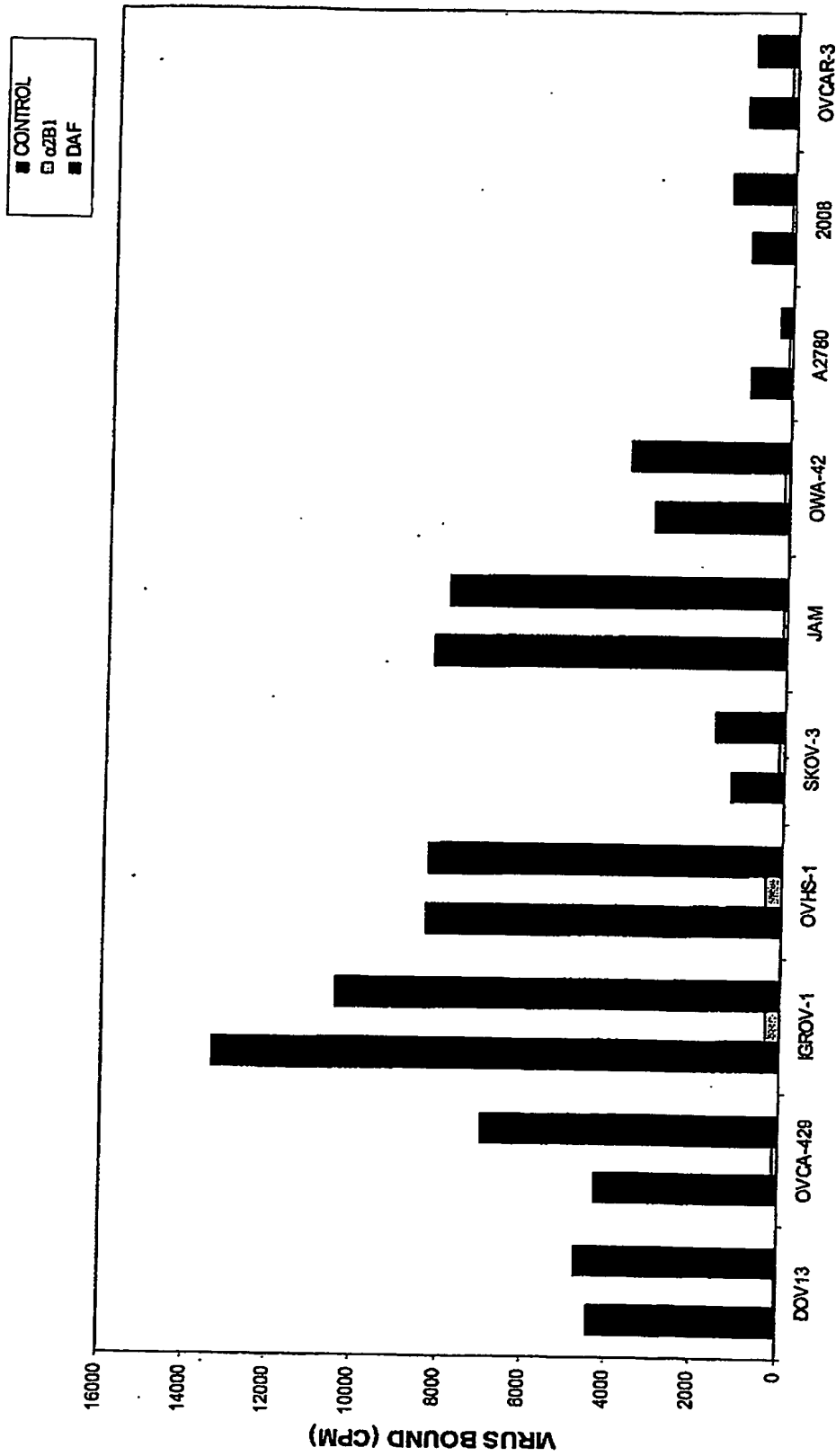


Fig. 11

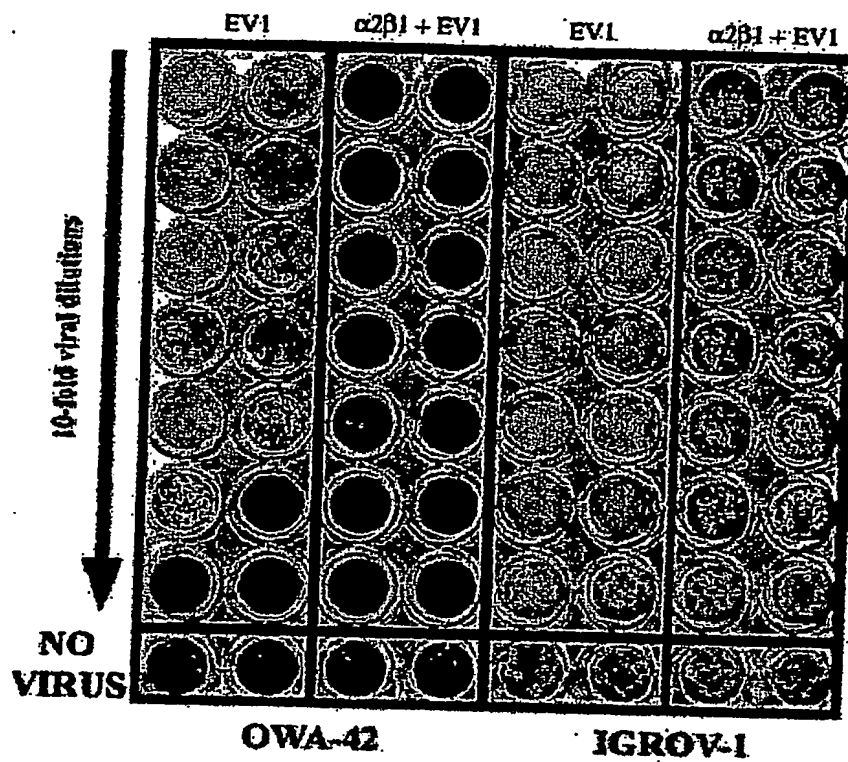


Fig. 12

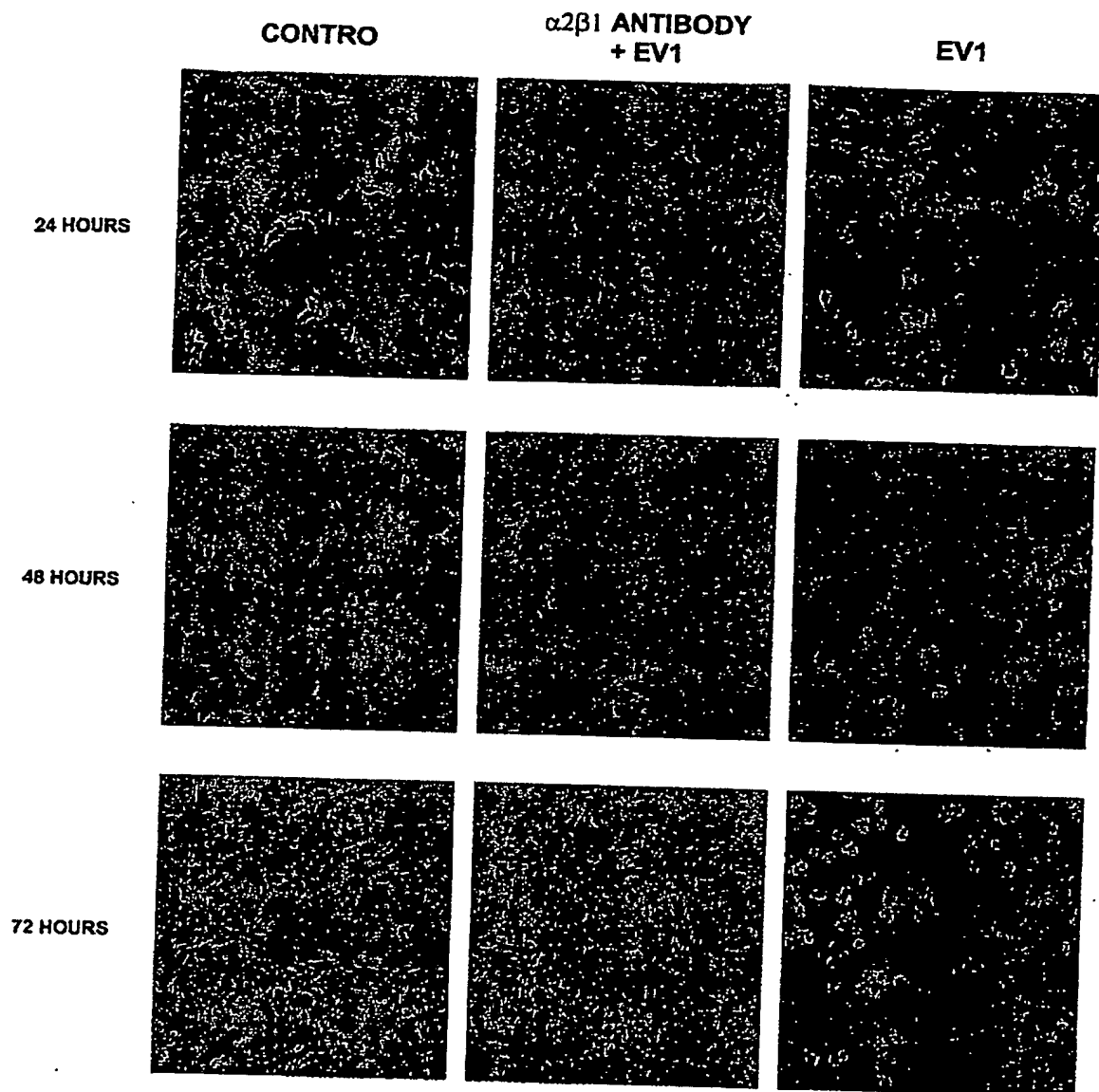


Fig. 13

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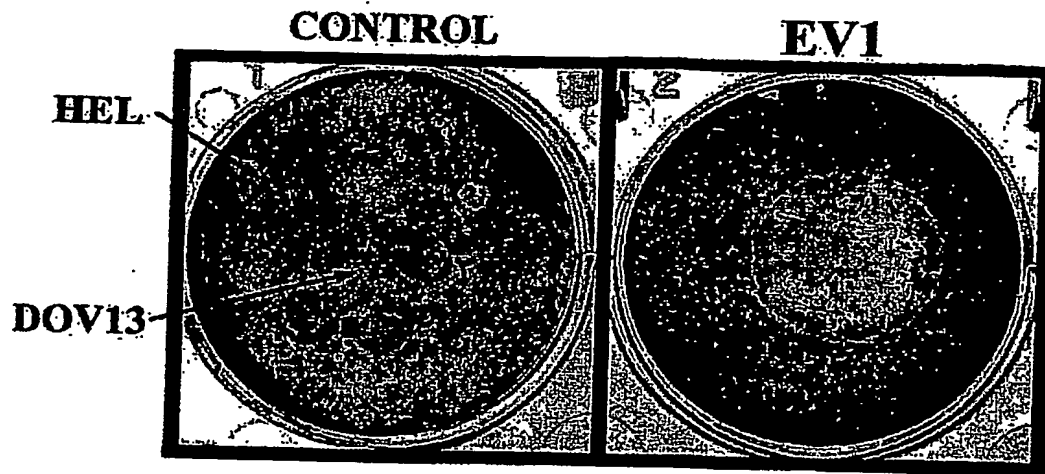


Fig. 14

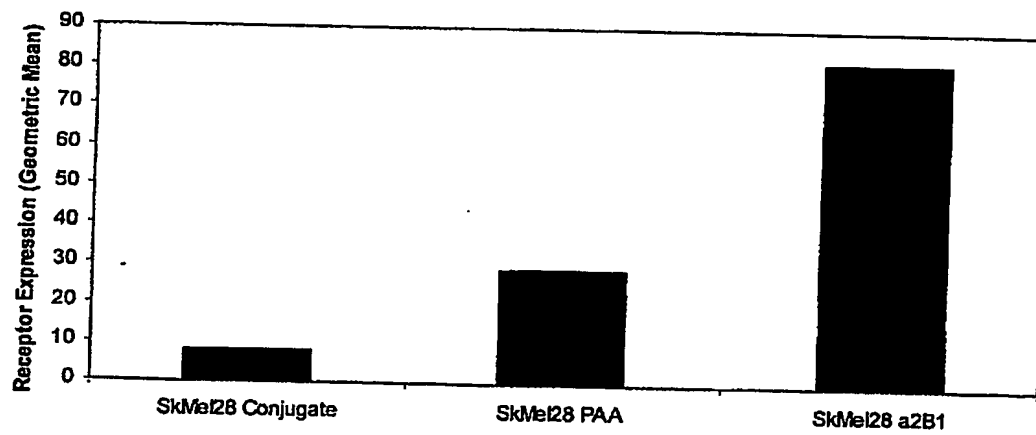


Fig. 15

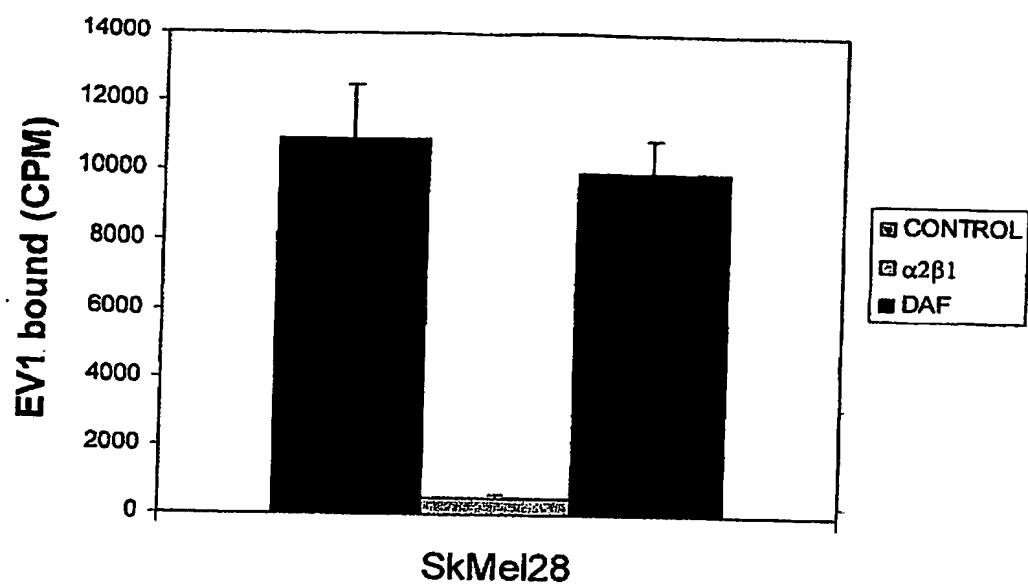


Fig. 16



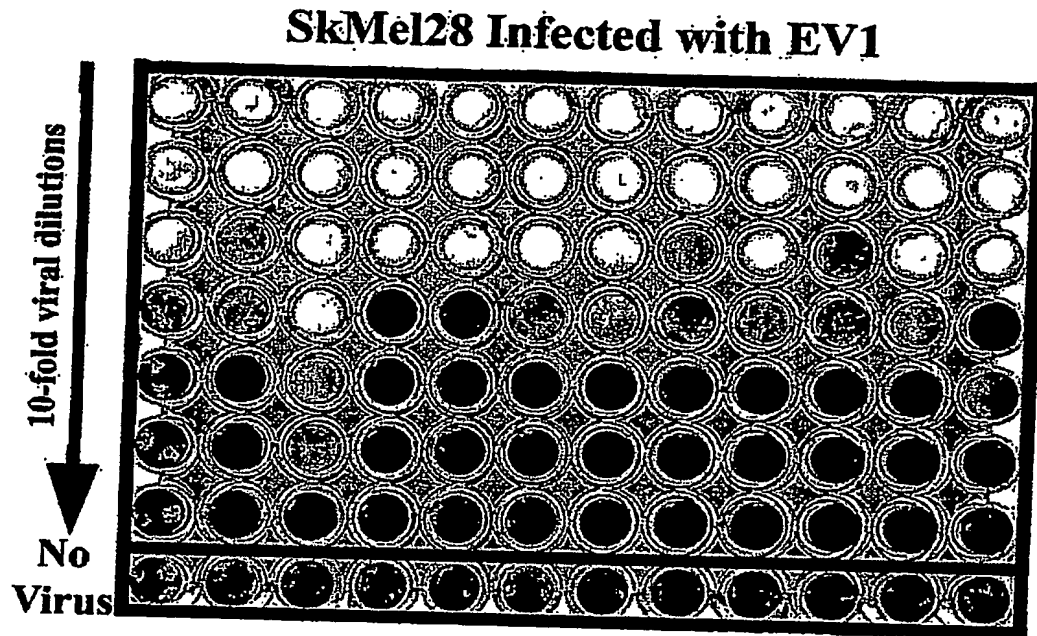


Fig. 17

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